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Proceedings of the Symposium on Experimental Models for Pulmonary Research

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Judith A. Graham

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Proceedings of the Symposium on Experimental Models for Pulmonary Research

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Foreword

Chronic and acute diseases are among the major causes of disability and death in all age groups. As the organ responsible for gas exchange, the lungs have a unique proximity to the environment and as such are repeatedly exposed to potentially hazardous gaseous and particulate airborne pollutants as well as microorganisms. In order to adequately assess the total impact of environmental pollutants on the health of human lung, it becomes necessary that disciplined studies with various animal model systems be available.

During the past several years a number of new sensitive and reproducible in vivo and in vitro pulmonary research model systems have been developed for studying the unique features of the respiratory tract, particularly those related to host defense mechanisms and pharmacological modulations in the lung. This symposium has been organized to exchange research ideas and techniques with experts in the scientific community and to explore the possible application of these new model systems and techniques for studying environmental effects on pulmonary health. The conference was a framework which can insure that the U.S. Environmental Protection Agency is kept up-to-date on the latest research developments in these areas.

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Edward P.C. Hu
Judith A. Graham

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Session I:
Permeability of Respiratory Epithelium

Chemical Modulation of Airway Epithelial Permeability

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Epithelia generally exhibit two functions in organ homeostasis: they serve as a protective barrier, and they often possess specialized mechanisms of transport. As the control of ion and mucus secretion by the airway mucosa will be discussed later in this symposium, this presentation will focus on the role of airway epithelium as a barrier to passive translocation of large polar solutes, both as inhaled materials and resident macromolecules, across the airway. Previous work on the "barrier function" of the airway mucosa, predominantly morphologic, will be reviewed, and more recent approaches to the study of airway epithelial function that combine physiologic measures of airway permeability and morphology will be presented.

Until the late 1960s, it had been held that the respiratory epithelium was impermeant to protein macromolecules. Morphologic studies reported by Schneeberger-Keeley and Karnovsky, employing horseradish peroxidase (HRP), a glycoprotein of ~40,000 daltons, as an electron microscopic tracer, suggested that in the mammalian alveolus macromolecular flow across the epithelium was completely restricted at the level of the tight junctions adjoining alveolar pneumocytes (22). However, physiologic studies initially reported by Bensach *et al.* suggested radiolabeled albumin crossed the alveolar epithelium at sites that were not defined (1). These findings were confirmed by others (25). Little information on the movement of macromolecules across airway mucosa was available until 1973 when Richardson *et al.* reported on attempts to localize within the airway wall inhaled protein antigens that elicited acute

allergic bronchoconstriction in sensitized guinea pigs (21). These authors were unable to detect antigen, in this case HRP, below the epithelial tight junctions at times coincident with physiologic airway constriction. A hypothesis advanced for these results suggested that the rapid onset of bronchoconstriction (~ 30 sec after initiation of challenge) involved antigen contact with mast cells residing in the airway lumen. Subsequent transmission electron microscopic (TEM) studies by Richardson *et al.* suggested that enzymatically intact HRP and ferritin do cross the guinea pig tracheal epithelium from airway lumen to interstitium (20). They concluded that this movement occurs via pinocytotic rather than paracellular routes, required at least 30 to 60 minutes for transfer, and no preferential rates of movement of HRP (40,000 d) over ferritin (450,000 d) could be shown. Inoue and Hogg in subsequent studies concentrated on defining the ultrastructure of the epithelial tight junctions (13). Freeze fracture studies from guinea pig trachea revealed a junctional compartment that is relatively deep-- ~ 0.5 microns--and composed of an average of 7 strands, interconnected to form compartments.

Studies of respiratory epithelial permeability to macromolecules have been hampered by several technical problems. Satisfactory probe macromolecules for use in estimating the permeability of relatively tight or impermeable epithelia have been lacking. Radioactive label from externally labeled probes often elutes or is cleaved from the parent compound. Because of the restrictive sieving characteristics of respiratory epithelia, flows of the lower molecular weight-free label or labeled fragments across the barrier will dominate and yield erroneously high permeability coefficients. In the worst cases, free label can bind to resident macromolecules, e.g., albumin, making evaluation of the nature of the permeant radioactivity by common techniques, e.g., TCA precipitation, impossible. Such considerations have similarly hindered combined morphologic-physiologic studies of macromolecular translocation in respiratory epithelia. Techniques such as autoradiography have been relatively disappointing for these purposes because there is little evidence that radioactivity faithfully reflects location of probe molecules.

These considerations led to the development of sensitive techniques to monitor the movement of the EM tracer horseradish peroxidase across relatively impermeable barriers. Because of the need to use small amounts and low concentrations of this material on mucosal surfaces, an assay sensitive to nanogram/ml quantities of HRP in plasma was required. As the presence of circulating endogenous peroxidases rendered the enzymatic determination of HRP in plasma at low concentrations unreliable, a solid phase radioimmunoassay (RIA) that detects HRP in plasma to concentrations of 10 ng/ml was developed. Following application of small volumes of HRP onto the airway

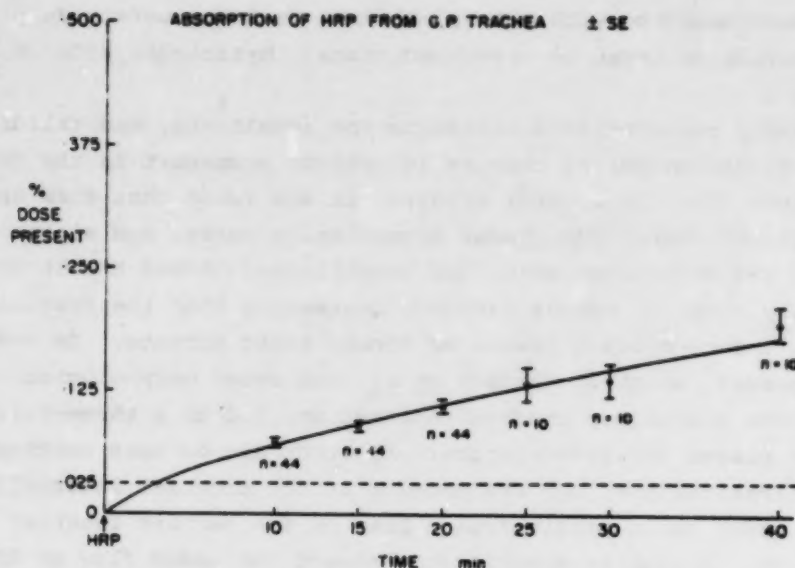


Figure 1. Plasma accumulation of HRP after intratracheal instillation of 1 mg HRP. Ordinate: fractional plasma absorption of instilled HRP; abscissa: time.

mucosal surfaces, rate constants of HRP transfer into blood can be estimated and routes of HRP movement across the epithelium examined morphologically with the transmission electron microscope.

Early studies showed that after instillation of 1 mg of purified HRP into guinea pig trachea a small fraction of the instilled HRP was measurable in plasma 10 minutes later (0.08% instilled dose) and that the rate of HRP accumulation in plasma was relatively constant from 10 to 40 minutes (Fig. 1). To assess effects of an inspired agent on tracheal permeability, the following protocol was used: After control blood samples were obtained, 1 mg of purified HRP in 0.2 ml phosphate buffered saline (PBS) was instilled onto the tracheal surface through a tracheostomy tube. Blood was sampled for HRP by RIA at 10, 15, and 20 minutes, with the rate of accumulation in plasma over this interval calculated by the least squares method. After the 20-minute measurement, an inhalation exposure to a test agent was administered and post-challenge rates of HRP accumulation in plasma determined from 20- to 40-minute measures of plasma HRP. The significance of aerosol-induced rate changes was estimated by comparing these rates for individual animals within each group with paired t-tests. At various points within this time frame animals were sacrificed and HRP localized in mucosal tissue on transmission electron micrographs. In addition, respiratory parameters, tidal volume, dynamic compliance, and pulmonary resistance, as well as arterial blood gases and

blood pressure, were measured with the animal in a pressure-sensitive plethysmograph to provide an index of more traditional physiologic effects of the inspired agent.

We have recently reported data assessing the sensitivity and validity of this methodology for measuring changes in protein transport in the tracheobronchial mucosa (7). In control studies, it was found that sham challenge with air did not change HRP plasma accumulation rates, and electron micrographs at 23 and 40 minutes after HRP instillation showed HRP to be localized primarily atop the mucous barrier, suggesting that the instillation technique did not induce mucosal trauma or direct toxic effects. In contrast, diethyl ether exposure, which Richardson *et al.* had shown morphologically to HRP penetration into guinea pig tracheal epithelium, led to a three-fold increase in rate of plasma HRP accumulation. Electron micrographs confirmed Richardson's observations that HRP was present in the proximal intercellular spaces, a finding that could result from a loss of the barrier function of the tight junctions. A similar association between increased flow of HRP from lumen to blood *in vivo* and loss of the barrier function of the tight junction has been reported in the gut (19). Gel filtration of plasma from control and exposure animals showed the HRP measured in plasma by RIA to be intact 40,000 d protein with no smaller molecular weight fragments measurable.

More recently, in collaboration with Ms. Joy Johnson in Montreal and Dr. Hogg, these techniques were used to pursue earlier morphologic observations of Simani *et al.* that inhalation of cigarette smoke increases HRP penetrance into guinea pig epithelium (14, 23). In these studies, guinea pigs, 20 minutes after intratracheal instillation of HRP, were assigned to one of five exposure groups: 1) a control group exposed to 20 puffs of air; 2) a group exposed to 5 puffs of whole cigarette smoke; 3) a group exposed to 20 puffs of whole smoke; 4) a group exposed to 100 puffs of whole smoke; and 5) a group exposed to 100 puffs of smoke that had been passed through a standard Cambridge filter pad, effectively removing the particulate phase of smoke and thus exposing the animal to the "vapour" phase of smoke. Smoke or air was delivered to the animals by a specially designed smoking machine that, from standard flue cure cigarettes, delivered 20-ml puffs of smoke into a length of tubing attached to the inspiratory port of the tracheostomy tube. The results for control, post-exposure rates, and p values for these groups are shown in Table 1. For the control group the mean rate of HRP accumulation after air exposure, while moderately lower, did not differ statistically from the control (10-20 minutes) period. Five puffs of whole smoke have relatively little effect on rates of HRP accumulation, while the mean rate of plasma HRP accumulation after 20 puffs of smoke was moderately increased, principally due to a variable effect at 40 minutes. It can be seen that 100

Table 1. Rates of Plasma HRP Accumulation for Control Periods and After Exposure to Graded Doses of Cigarette Smoke

	(5% delivered dose/min)		p	$\frac{20-40}{10-20}$
	10-20	20-40		
Control (N=14)	0.0053	0.0038	NS	0.7
5 puffs (N=12)	0.0055	0.0053	NS	1.0
20 puffs (N=11)	0.0055	0.0099	0.09	1.8
100 puffs (N=15)	0.0048	0.0122	<0.01	2.5

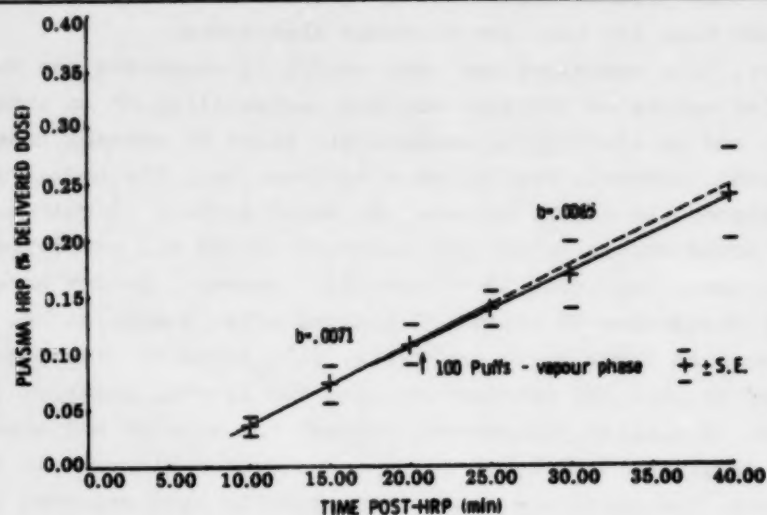


Figure 2. Rate of plasma HRP accumulation before ($b = .0071$ percent delivered dose min^{-1}) and after ($b = .0065$) exposure to vapour phase of cigarette smoke.

puffs of whole cigarette smoke resulted in about a three-fold rise in the rate of plasma HRP accumulation over control, an increase significant at the $p < 0.01$ level. In addition to standard transmission electron microscopic studies, freeze fracture studies were performed by Sada Inoue on tracheal epithelium from guinea pigs that inhaled equivalent amounts of smoke. In contrast to the normal morphology as revealed by this technique, beading and disruptions of the fibrils, loss of discrete compartments, and free fibrillar endings were noted after smoke inhalation. While the precise relationship

between the morphology of the junction as revealed by freeze fracture techniques and probe permeation is controversial, the changes are consistent with the TEM evidence of damaged tight junctions and the increases in probe (HRP) flow.

Figure 2 shows the application of these techniques to identify the noxious components of cigarette smoke. This figure shows that exposure to 100 puffs of the vapour phase of cigarette smoke--that is, whole smoke passed through a Cambridge filter--is not associated with increased rates of plasma accumulation of HRP over control. These data suggest that the tars and nicotine in the particulate phase rather than the acroleins, CN^- and NO_x in these vapour phase exposures, are likely to be the active agents. Data consistent with these observations have recently been obtained, showing that high tar, high nicotine cigarettes produce significantly greater increases in tracheal permeability to HRP than low tar, low nicotine cigarettes.

In summary, this technique has been useful in measuring the changes induced by inhaled agents in tracheal mucosal permeability to an intact macromolecule, HRP, and in identifying morphologic sites of mucosal damage.

The protocol, however, has raised questions about the normal routes of HRP movement across the airway mucosa. As noted earlier, Richardson *et al.* suggested HRP moves across guinea pig tracheal mucosa via pinocytosis relatively slowly, i.e., requiring 30-60 minutes. However, in our experiments HRP was easily measurable in plasma 10 minutes after instillation. Electron micrographs taken at these early intervals, like those of Richardson and co-workers, failed to show HRP penetration into the lateral intercellular spaces or basal areas. A similar discrepancy between the rate of HRP movement across the pulmonary capillary to R lymph duct, and electron microscopic evidence of tracer egress from capillary lumen, has recently been reported (2). These observations imply that standard EM techniques, not sufficiently sensitive to detect a small number of HRP molecules traversing the tight junction, are unable to detect transport via a small number of vesicles emptying into the lateral or basal areas of the epithelium. Alternatively, localized areas of transport such as the bronchus associated lymphoid tissue (BALT) (16) areas or increased cell turnover may be missed by the sampling procedure.

To better describe the movement of macromolecules across airway epithelium, precise measurement of translocated probes and the surface area available for permeation is necessary. *In vitro* studies with excised trachea, mounted in Ussing chambers, as first reported by Olver *et al.*, have proved useful for such studies (17). In these experiments, epithelium from the posterior membrane of canine trachea is mounted as a flat sheet between two Lucite half chambers. The bathing solutions, generally Ringer's or Krebs-Henseleit, are gassed, circulated, and warmed. In most situations, these solutions are identical on both sides of the membrane, eliminating chemical

Table 2. In Vitro Bioelectric Properties of Excised Canine Trachea
in Ussing Chambers

	Bioelectric properties ^a	
	Present study	Olver <u>et al.</u>
PD (mV)	33.5 ± 2.9	30.7 ± 2.7
I _{SC} (μAcm ⁻²)	97.6 ± 11.0	108 ± 8.0
G (mScm ⁻²)	2.91 ± 0.18	3.51

^a Mean ± S.E.

and hydrostatic pressure gradients. Via sensing bridges closely apposed to each surface of the membrane, transmural potential difference (PD) is recorded with a voltmeter. The membrane can be short-circuited, that is, the trans-epithelial PD reduced to zero, by imposing the electromotive force (EMF) of an external battery across the membrane via a second pair of bridges. The current through the external circuit short circuit current (I_{SC}) is recorded on an ammeter. Because this tissue behaves like an ohmic resistor, resistance (R) and its reciprocal, conductance (G), can be calculated from the I_{SC} and open circuit PD. For our studies, probe molecules, which differed in size but too large to enter the intracellular space, were added to the mucosal bathing solution and the rate of probe appearance in the serosal solution was monitored by tracer counting (¹⁴C-mannitol, ³H-inulin) or RIA (HRP) (4). Permeability coefficients for these probes were calculated.

The bioelectric properties of canine trachea measured in these studies were similar to those reported by Olver et al. (17) (Table 2). Probe molecule addition, particularly HRP, at a dose of 7.5 mg/ml, did not change bioelectric properties. Rates of entry into the sink were linear 30 minutes after the molecules were added to the source.

From the data of Marin et al., it can be calculated that the electrical G (3.3 mScm⁻²) approximates the sum of J_{Cl}^{M-S} (2.4 mScm⁻²) and J_{Na}^{S-M} (1.4 mScm⁻²)--i.e., I_J^{passive} = 3.8 mScm⁻² (15). Further, permeability coefficients for Na⁺ and Cl⁻ can be calculated from these fluxes. The ratios of P_{Cl} and P_{Na} to P_{man}--3.2 and 1.6, respectively--approximate the aqueous free diffusion ratios: D_{Cl}⁻/D_{man} = 2.8; D_{Na}⁺/D_{man} = 1.9. Because mannitol is assumed to move through extracellular paths, the similarity in the permeability ratios and the ratio of the free diffusivities suggest passive Na and Cl movement is unrestricted and occurs largely via aqueous paracellular routes.

The permeability coefficients of the respiratory mucosa in vitro to probe solutes, the ratios of P_{probe} to P_{mannitol} and free diffusion ratios of

Table 3. Permeability of Canine Tracheal Epithelium to Solutes
in Vitro

Probe solute	Canine trachea		Free diffusion
	P ($\times 10^7$ cm sec $^{-1}$)	$P_{\text{probe}}/P_{\text{mannitol}}^a$	$D_{\text{probe}}/D_{\text{mannitol}}$
$^{36}\text{Cl}^-$	52 ^b	3.2	2.8
$^{22}\text{Na}^+$	25 ^b	1.6	1.9
^{14}C -mannitol	16	1	1
^3H -inulin	4.4	0.25	0.33
Horseradish peroxidase	0.1	0.006	0.076

^aCalculated from the contemporaneous fluxes of mannitol and the probe across the same preparation (except for Na^+ and Cl^- permeation through dog trachea).

^bCalculated from the lumen to serosa flux of Cl^- and the serosa to lumen flow of Na^+ reported by Marin and coworkers.

D_{probe} to D_{mannitol} are shown in Table 3. The comparison of permeability coefficients is one of the methods used by Solomon and others to calculate equivalent pore radii (24). In this analysis $P = F(a/r) \frac{D}{\Delta X}$, where

$$F(a/r) = (1 - a/r)^2 (1 - 2.1 a/r + 2.09 a/r^3 - .95 a/r^5) \text{ and}$$

a = probe molecule radius; r = pore radius; and ΔX = path length.

Comparison of the permeability coefficients for probe molecules to a common reference, i.e.,

$$\frac{P_X}{P_{\text{ref}}} = \frac{F(a_X/r)}{F(a_{\text{ref}}/r)} \cdot \frac{D_X}{D_{\text{ref}}}$$

eliminates the path length. The results of such an analysis are shown in Figure 3. As the molecular radius of the probe molecule increases, the permeability coefficient, relative to that of mannitol, decreases. The direction of this change is predicted for movement in free solution. However, the ratios for inulin and, particularly, HRP are smaller than those predicted for free diffusion, suggesting that the movement of these molecules is restricted and that the barrier behaves like a "sieve." A single equivalent pore of 75 Å appears to fit our data. Although this pore size may seem relatively large in light of published comments on the relationship between PD, conductance, and the "tightness" of epithelia (10), the total pore area can be calculated to occupy 0.05% or less of total surface area. The presence of albumin, a macromolecule similar in size to HRP, in canine airway liquid (18) obtained *in vivo* from normal dogs suggests that these paths are present under physiologic conditions.

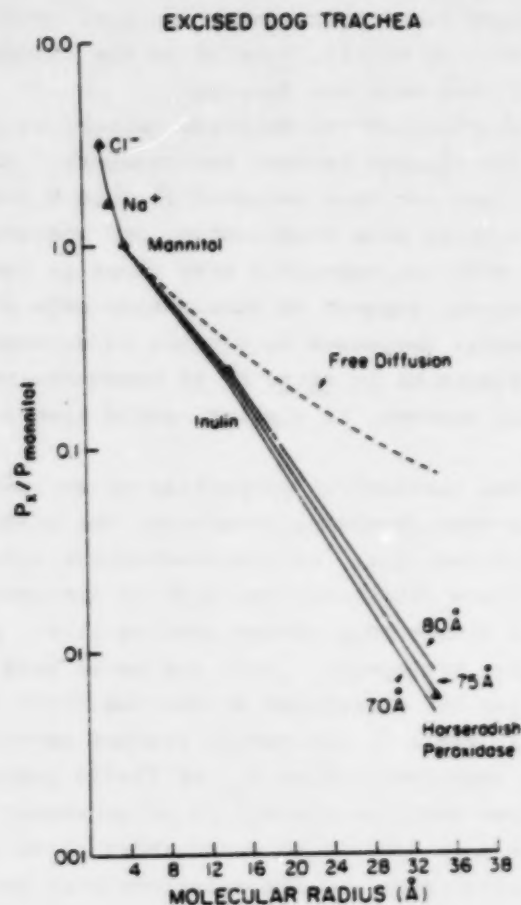


Figure 3. Semilogarithmic plot of ratio of $P_{\text{probe}}/P_{\text{mannitol}}$ versus molecular radius. Solid lines show predicted values for equivalent pore radii 70-80 Å.

It should be noted that the electrical conductance (G) reflects current that is carried by small ions. The magnitude of the conductance may reflect the total area of the aqueous channels in the barrier, but the magnitude of restricted diffusion or apparent pore dimensions cannot be predicted directly from G.

The existence of size-dependent restriction of "sieving" solutes across tracheal epithelium implies movement through paracellular pores rather than by pinocytosis. Other evidence supports this hypothesis. The rate of flow of probes was directly related to tissue conductance, indicating movement of these solutes by the same paracellular paths as ions. The unidirectional

fluxes of the probes were symmetric, i.e., flow from S-M equaled flow from M-S, as would be anticipated for a diffusional process. Finally, in collaboration with Dr. N. S. Wang of McGill, samples of the tissues were examined ultrastructurally and vesicles were not detected.

Edge damage, i.e., chamber-induced membrane damage, is a concern in all experiments where tissue is clamped between two chambers. Although rigorous analyses of edge effects have not been reported in this tissue, varying edge to surface area ratios, varying edge compression, and comparison of bioelectric properties obtained with our apparatus with those in the literature, including "edge free" systems, suggest no substantial edge artifacts in these experiments. As edge-induced increases in G might be expected to decrease PD measured in vitro, comparisons of in vitro PD to measurements of PD made directly in vivo in canine trachea, if similar, would also suggest no substantial edge artifacts.

As reviewed above, the bioelectric properties of excised canine trachea can be measured in Ussing-type chambers, permitting the study of the tissue under short circuit conditions, i.e., no electrochemical driving force for solute movement. Under these conditions and with no hydrostatic gradient, asymmetric unidirectional fluxes of a tracer species (i.e., net flow) are primary evidence for active transport. Olver and Marin have demonstrated Cl^- secretion and a smaller Na^+ absorption across the short circuited canine trachea (17). As shown in Table 1, the canine trachea exhibits an average peak PD of ~ 30 mV--lumen negative--and an I_{SC} of $75-100 \mu\text{amps/cm}^2$. The radio-tracer studies suggest that the open circuit PD is generated by active ion transport mechanisms that were identified under short circuit conditions. Several pharmacologic agents, added to the solutions that bathed canine trachea in vitro, have been shown to alter bioelectric properties and ion fluxes.

However, the relevance of the in vitro model to in vivo function, both for our studies and salt and water metabolism, hinges on the similarities in the basic properties of the two preparations. The selection of appropriate bioelectric properties for comparison is not obvious since the time course of PD in vitro is complex. After mounting, transepithelial PD transiently falls but subsequently gradually rises to reach a relatively stable plateau 1-2 hours after mounting. We and others have selected this plateau value as the value likely to represent the PD of the epithelium in vivo. To verify this supposition and because Hale et al. have reported an in vivo canine tracheal PD of only -5 mV, we have developed a technique to measure PD directly in vivo (3, 12).

The protocol is begun by balancing the reference electrode--a 19 gauge needle containing Ringer-4% agar--and the fluid-filled exploring bridge (PE-160 tubing) in a common reservoir of Ringer. The calomel electrodes connect-

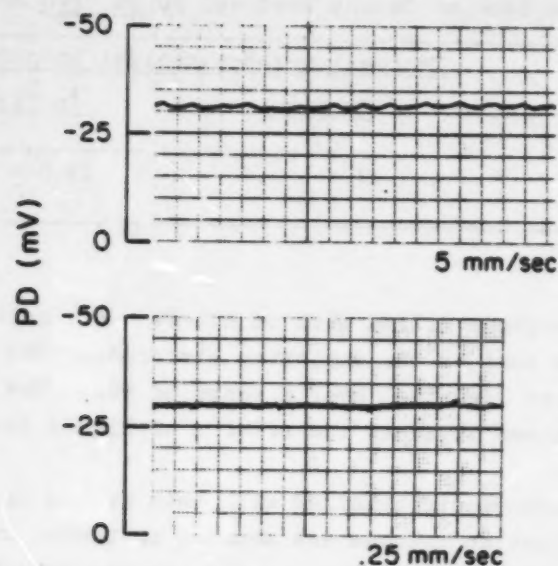


Figure 4. In vivo PD (mV) recordings from canine trachea.

ing these bridges to the voltmeter were selected to have an offset potential of 1 mV for each study. Adult male mongrel dogs, mean weight 18 kg, were anesthetized with intravenous pentobarbital sodium or amybarbital and intubated. The reference electrode was placed in a subcutaneous space, shown in preliminary studies to be isoelectric with the serosal surface of the trachea. The exploring electrode was advanced through the endotracheal tube. Contact with the tracheal surface was insured by continuous perfusion through the bridge at 0.1 ml/min with warmed, gassed Ringer solution. The potential sensed by this bridge was transmitted to the calomel half-cell via Ringer-4% agar bridge that connected the perfusion reservoir with the cell.

The calomel electrodes were connected to a high impedance voltmeter--filtered to remove 60 cycle interference--and values displayed digitally and recorded on a strip chart.

Figure 4 shows representative tracings obtained with the fluid-filled bridge. It can be seen from the top tracing--with relatively fast paper speed--that the PD recorded from this dog is stable but shows oscillations, which correspond to respiratory movements. The bottom tracing shows a 6-minute interval, indicating stability of tracing. Continuous tracings are stable, within 10%, over periods of at least 30 minutes.

To control for possible effects of liquid junction potentials between bridge perfusate and tracheal liquid, 3M KCl bridges were compared to the Ringer bridges employed in the fluid-filled system. In these experiments a 3M KCl-4% agar-filled 19 gauge needle was inserted into a subcutaneous

Table 4. Comparison of Canine Tracheal PD in Vivo and in Vitro

	Tracheal transepithelial biopotential ^a	
	<u>In vivo</u>	<u>In vitro</u>
PD (mV)	29.1 $\pm$ 3.0	33.5 $\pm$ 2.9

^aMean $\pm$ S.E.

space and a PE-160 electrode filled with 3M KCl-4% agar, balanced as discussed previously, was used as the exploring electrode. The values obtained with each technique were identical over a range of PDs. The correspondence of PD with the two systems suggests the effects of liquid junction are negligible.

For comparing measurements obtained with this system directly to in vitro measurements, excised canine trachea was mounted in Ussing chambers and PD recorded by standard techniques. Then the chamber was detached from the in vitro apparatus, the reference 19 gauge needle electrode inserted into the solution bathing the serosal surface of the membrane, fluid evacuated from the mucosal side, and PD recorded directly by introducing the fluid-filled exploring electrode onto the mucosal membrane surface. The potentials measured with in vitro and in vivo bridges in this situation were identical (n=6). These data suggest that the filtering techniques used to process the in vivo signals did not contribute to the measured potentials and indicate a close correspondence between in vitro and in vivo measured PD in the canine trachea.

Further, we have compared PDs measured with the fluid-filled bridge in other organs with those reported in the literature. Canine gastric PDs from three dogs were measured under fluoroscopic control. The mean PD, -54 mV, is similar to that reported by Dennis and others (9). Esophageal PDs in these dogs were -20 mV, with a regional PD profile similar to that reported in rabbit and man by Powell and coworkers (26).

PD was measured at three random sites in the trachea and mean value calculated. Table 4 shows the mean PD $\pm$ S.E. from 27 separate dogs using the FF technique and, for comparison, the mean peak in vitro PD $\pm$ S.E. from the study previously cited from our laboratory. Mean tracheal PD is -29 mV, a value indistinguishable from peak in vitro values.

The fluid-filled bridge has made it possible to assess the pharmacologic modulation of PD in vivo by direct addition of agents to the bridge perfusate, ensuring application of drug directly at the site of PD measurement. Drug-containing Ringer is flushed through the bridge perfusion system pump run in reverse for 1.5 minutes. During perfusion the time required to clear the drug-free fluid provides a control period. Drug effects were compared to

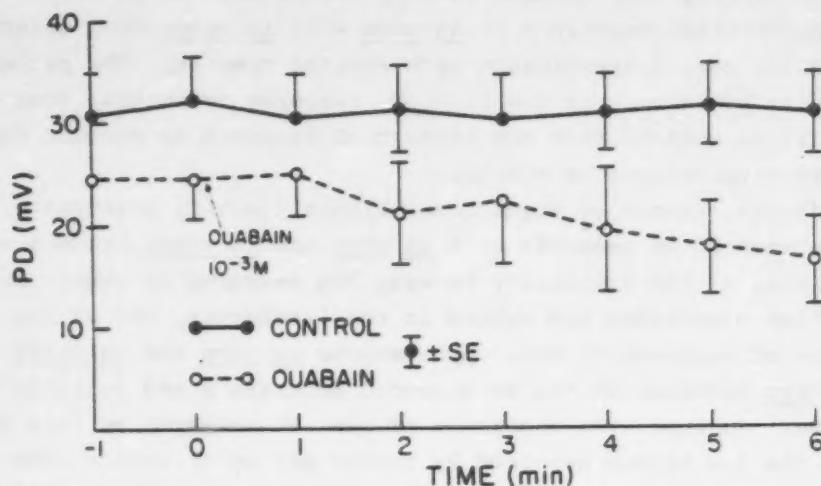


Figure 5. PD response to ouabain addition to mucosal surface of canine trachea.

Table 5. Comparison of in Vivo and in Vitro PD Response in Canine Trachea to Mucosal Applications of Pharmacologic Agents

	Profile of PD response ^a	
	<u>In vivo</u> ΔPD (mV)	<u>In vitro</u> ΔPD (mV)
Ouabain (10 ⁻³ M)	-33.5%	-28.0%
Amphotericin (10 ⁻⁵ M)	+77.0%	+58.0%
Acetylcholine (5 x 10 ⁻⁴ M)	0	0
Atropine (10 ⁻³ M)	0	0
Histamine (10 ⁻³ M)	-10.0%	+5.0%

^aPercentage control.

those of a group of 10 dogs with Ringer perfusion only. An example of the effect of ouabain, 10⁻³M, on tracheal PD in four dogs is plotted as a function of time in Figure 5. Ouabain produces a slow reduction in PD. Both the magnitude of the change and time course resemble those reported for the in vitro preparation.

As the majority of the reports on pharmacologic perturbation of tracheal bioelectric properties have focused on only the effects of serosal drug addition, an appropriate comparison of in vivo with in vitro drug effects required a parallel set of experiments with excised trachea. The pattern of in vivo response is compared with the in vitro response to mucosal drug additions in Table 5. It is obvious that the pattern of response to mucosal drug application in both preparations is similar.

Hence, 1) the absence of significant liquid junction potentials, 2) the similarity between PD as measured with in vivo and in vitro bridges on excised epithelium, 3) the similarity between PDs measured in other organs with the fluid-filled electrodes and values in the literature, and 4) the similarity in pattern of response to drug applications in vivo and in vitro indicate that the in vivo tracheal PD can be measured accurately and reliably with this technique. Further, the magnitude of that PD measured by this technique implies that the low values reported by others may be in error. The technique should allow direct in vivo studies of the genesis and regulation of the airway biopotential in both animal and man. In regard to the interests of this symposium, effects of environmental pollutants on airway potentials in vivo are feasible.

There are several likely pathophysiologic implications of airway hyperpermeability. A loss of the "barrier" function would facilitate movement of inhaled materials into the airway wall. Recent studies in conjunction with Dr. J. Richardson at McGill have shown in the chicken that airway hyperpermeability, induced by methacholine, enhances antibody response to antigen instilled into the trachea (unpublished data). This enhanced response was assumed to result from an increased antigen load. The effects of airway hyperpermeability on airway reactivity in asthmatics to naturally occurring antigens have not been evaluated. However, the hyperreactivity to inhaled chemical agonists that is a characteristic of asthmatics may, in part, be mediated by airway hyperpermeability.

It has been shown in the ascaris-sensitive rhesus monkey and guinea pig that specific antigen challenge leads to mucosal hyperpermeability to topically applied small molecular weight polypeptides and HRP (5, 6). Further, the rhesus demonstrates a period of hyperreactivity to inhaled histamine for an interval after antigen challenge, even after R_L has returned to baseline. Using radiolabeled histamine as a tracer, it has been observed that post-challenge airway hyperreactivity is associated with an increased fractional absorption of inhaled histamine into the blood (8). These data are consistent with the hypothesis that airway epithelial hyperpermeability, induced by allergic bronchoconstriction, contributes to airway hyperreactivity by increasing flows of inhaled bronchoactive agents to effector sites in the airway wall. However, until experiments can be performed that alter airway

permeability in the absence of changes in bronchomotor tone, the association of airway hyperpermeability and hyperreactivity cannot be taken as causal. The recent demonstration, in collaboration with Dr. Richardson, that viral infection in chickens increases airway permeability to HRP suggests that this mechanism may contribute to the increased airway reactivity with viral infections in humans.

A hyperpermeable mucosal surface might be expected to impair mucus clearance by two mechanisms. First, if active secretion of chloride secretion into the airway lumen is important to hydration of the airway, epithelial hyperpermeability, by increasing the passive "leak" of the chloride toward the interstitium, dissipating the osmotic gradient that drives water toward the lumen, would be expected to result in dehydration of the airway. Second, increased flows of macromolecules, e.g., albumin, through a more permeable epithelium down the chemical gradient into the airway lumen could occur, as has been reported for albumin in asthmatics (11). Albumin binds to mucus glycoproteins, increases cross-linking, and may thereby alter visco-elastic properties and diminish mucociliary clearance.

SUMMARY

The epithelium lining the airway appears to function as an effective, though not absolute, barrier to the movement of large hydrophilic substances between airway lumen and interstitium. The principal path of translocation for these substances across the airway mucosa appears to be paracellular. Experiments on the guinea pig are consistent with the notion that a variety of inhaled noxious agents, e.g., ether and cigarette smoke, increase epithelial permeability to HRP by increasing the flow of this probe through paracellular paths, probably by damaging the epithelial tight junctions. In vivo tracheal PD in dogs agrees closely with in vitro values and suggests the use of this technique in rapid screening for epithelial damage.

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DISCUSSION

PARTICIPANT: Is there any difficulty that you can foresee in testing alterations of permeability in the in vitro preparation? In other words, couldn't you simply put ether into the in vitro preparation, and try to open up tight junctions, as you've mentioned, and see whether you get effects in conductivity or pore radius or whatever?

DR. BOUCHER: I think that kind of study may have a fruitful future. We're actually beginning to do cigarette smoke in a parallel fashion in vitro. I think the point is that you can't just measure conductance, if you're interested in what's happening as far as equivalent pore radius. But I think our data with respect to the in vitro preparations, are relatively encouraging. The potentials that we measured, and I think they were measured in San Francisco, are quite close to what we measure in vivo.

DR. DAVIS: I'm glad that you do find the same; it seems that our last five years of work haven't been in vain. I do have one comment and one question. Originally, we measured in vivo versus in vitro potentials, but we did it a little bit differently. We took a segment of the trachea from a dog and coated just the top half of it with fluid. Then we planted the lower half of the trachea from the same dog in the chamber. We found a correlation between the two, but we haven't published the results. We now, in fact, have a chronic dog with emphysema and from the fluid-filled pipe we measure PD, and from day to day in a chronic dog, that seems to be a fairly standard contact.

DR. BOUCHER: Do you get 30 millivolts?

DR. DAVIS: In the chronic, it's not as high as that. It's around 12 or 15 millivolts. My question is whether you are using our original preparation? Do you dissect off the trachealis muscle, and have you done histological studies of that? One of our worries always was that sometimes there are glands there, and those glands run right up to where the muscle is. Then, there is the possibility that would cut through one of those glands and open up one of the gland ducts as a pore through the epithelium.

DR. BOUCHER: There may be large leaks but we have not found them despite large numbers of histologic studies. We actually had a more troublesome worry

in regard that there were no pores infected. Essentially we did slides; we did not see a mucosal lens, although we are sure that they are probably there. There may be an occasional large leak that we're not picking up under this technique. Again, this is purely a descriptive way of handling the data. We chose a particular analysis, because it had certain theoretical advantages, but Stein also used a similar model. The problem that we ran into is this:

(Dr. Boucher refers to a slide.)

This is a piece of tissue from the Ussing chamber, and all these black shapes probably represent bronchosepticus. They are all gram-negative rods, and we also found polymorphonuclear and lymphoid cells in this tissue.

DR. DAVIS: Can you tell us a bit more about that tissue? Have you done a study on that tissue?

DR. BOUCHER: This tissue had approximately 34 millivolts, a pore radius exactly similar with the others, and a conductance of less than 2. Most of our conductances run a little more than 2 2.2. We then got a group of uninfected female beagles. I don't have a picture of their epithelia, but they were clean; there were none of these black agents in it. The mucosal cultures also were negative, and the equivalent pore radii were identical. The bioelectric properties, as well, were identical. Infected tissue is a real problem. However, controlling epithelium in uninfected dogs did not change our data at all.

DR. DAVIS: Can I also establish that's not what you normally see in your preparation? I mean, your normal preparation doesn't look like that. That's not representative of every tissue you put in the chamber, is it?

DR. BOUCHER: It's representative of most male mongrel dogs, as far as the infection. Our dogs are all infected. I suspect that most are. We looked at straight H and E sections from these preparations, and they looked fine; you don't see it with such techniques.

DR. DAVIS: We've done EM studies. I'll be showing some pictures; I don't have many pictures of canine trachea with me, but I have some of cat trachea.

DR. BOUCHER: To us this finding was amazing.

PARTICIPANT: In the first part of your studies, where you studied in vivo, how do you know that the passage is in the tracheal or the bronchial epithelium, rather than in the lower airways?

DR. BOUCHER: We instilled a very small volume of material--about 200 microliters--into the trachea, and with two techniques we tried to assess the deposition. We radiolabeled some HRP with ^{125}I , then cut serial sections and counted. We found no tracer in the alveolar surfaces. Perhaps more directly, we fixed the trachea and performed the peroxidase reaction directly in the airways. We could only find HRP in the trachea and bronchi. Finally, we evaluated tissue, both from bronchi and the trachea by ultrastructural techniques. We had such an unrewarding experience with the bronchi, i.e., finding positive HRP on the mucosal surface, that we gave that up and just examined tracheal specimens. I'm fairly confident that these data really reflect HRP permeation in the central airway.

DR. SPITZNAGEL: What is the basement membrane in a surface situation? What do you see when you look at the basement membrane underneath the epithelium?

DR. BOUCHER: It looks relatively intact. It's interesting to look at the symmetry itself of peroxidase from both the mucosal side and the serosal side. Peroxidase from the mucosal side is not seen on transmission electron micrographs, but when you add it to the serosal side, you see peroxidase accumulating in proximal intercellular spaces between the cells. You do not see it at all in the basement membrane, even though the concentrations in that situation are 7.5 milligrams per l. The supposition is that the basement membrane isn't a significant barrier, at least as assessed by this relatively gross technique in this preparation.

DR. COLLIER: On that last slide that you showed, the bacteria in the intercellular spaces are also granules within the epithelium itself.

DR. BOUCHER: Yes, there were some early changes in the epithelium. It is not normal. I believe I said it looked like a viral infection as noted in other epithelia and as seen in humans. I discussed it with our veterinarians in the School of Veterinary Medicine. Apparently all our dogs are infected with a parainfluenza virus and the super-infected with gram-negative organisms.

Chemical Modulation of Alveolar Epithelial Permeability

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The respiratory epithelium is the first continuous cellular barrier encountered by environmental agents in inspired air and is, therefore, a potential site of early toxicity. In contrast to Dr. Boucher's contribution, this paper will focus on the functions of the epithelium that lines the gas exchange rather than the conducting surface of the lung. The fluid that covers the surface of the alveolar epithelium plays an important role in inflation of the lungs during fetal development (30) and in the maintenance of reduced surface tension and macrophage function in the adult lung (37). The volume and composition of the fluid are regulated by the permeability and active solute transfer processes of the epithelial monolayer.

Direct measurement of fluid outflow from the trachea (17) and dilution of an impermeant solute that was placed in the alveolar lumen (23) demonstrated that lung liquid is continuously secreted by the lung of the fetal sheep. The driving force for fluid production is the active secretion of Cl^- and K^+ by the epithelium (25). Secretion ceases and fluid is reabsorbed at birth, a phenomenon that can be simulated by the parenteral administration of epinephrine and blocked by a β -adrenergic antagonist (40). The barrier separating blood and lymph from the fluid that normally fills the lumen of the fetal lung exhibits many properties of a "tight" epithelium. Lipophilic solutes permeate at a rate roughly proportional to their lipid solubility. In contrast, the translocation of hydrophilic molecules is restricted by molecular size. Conventional pore analysis indicates that the permeation of small nonelectrolytes is compatible with aqueous channels with an equivalent

radius of 0.55 nm (24). A similar analysis of solute flow between blood and lymph suggests pores of 15 nm in the capillary endothelium (24). Hence, the epithelium presents the major impediment to solute movement between blood and the lumen.

Relatively little is known about the composition of the thin fluid layer at the air-epithelial interface in the adult lung. Several lines of evidence indicate that surface active material is secreted into the alveolar lumen by type II pneumocytes (16, 18). Fluid from the surface of alveoli of rats has been sampled by micropipet and found to be "protein free" (27). When a Ringer solution is added to the airspace of dogs and rabbits, the pattern of passive solute permeation between blood and the lumen resembles, in most cases, that of the epithelium of the fetal lung (35, 36, 41). Similar conclusions can be reached from studies of the osmotic effectiveness of small, hydrophilic solutes added to the solution that perfused excised dog lungs (34). The introduction of a salt solution into the airspace greatly increased the osmotic activity of solutes in the vascular space. These results suggested that the epithelial barrier between the new fluid compartment and the blood was far less permeable than the endothelial barrier between blood and the lung interstitium. Other studies have shown that the apparent radius of pores in the epithelium can be greatly increased by excessive inflation (2).

Several conditions and toxic agents damage the barrier between blood and the airspace and induce pulmonary edema. In all cases, the electrolyte composition of the accumulated liquid resembles that of extracellular fluid, but the protein concentration is determined by the method of edema formation. For example, increased left ventricular pressure in dogs results in the formation of edema fluid with the protein concentration of the interstitium (39), and a bulk flow of fluid across the epithelium through a few large holes (3). Alloxan induces the flow of fluid with the protein concentration of the plasma through many smaller holes in the endothelial-epithelial barrier (22, 38). Both methods of edema formation induce peribronchial and perivascular cuffs (29). These studies illustrate a major drawback of fluid-filled lung preparations. The movement of solutes across the alveolar epithelium cannot be distinguished from parallel flow across the epithelium of the airways. Since the surface area of the alveoli exceeds that of the airways by at least several hundred fold, the contribution of the nonalveolar epithelium to results from whole lung is often dismissed. However, recent evidence suggests that equivalent pores in the epithelium of the upper airways are much larger than those of the alveolar epithelium so that most large hydrophilic molecule permeation may follow this path (1).

METHODS, RESULTS, AND DISCUSSION

We have attempted to elucidate the permeability of the alveolar epithelium and the mode of action of toxic agents which attack this barrier in two ways. The first approach proposes the excised rat trachea as a model for the airway epithelium. When the permeability and transport functions of this barrier are "subtracted" from properties of perfused fluid-filled rat lung, the characteristics of the alveolar epithelium should be obtained.

Perfused, Fluid-Filled Left Lobe of the Rat Lung

A complete evaluation of the mode of solute translocation and edema formation in fluid-filled lung is hampered by the overwhelming contribution of fluid in the airspace to the electrolyte and water content of the tissue. We minimized this problem by preparing lung slices from a perfused lobe (32). The left lobe of the rat lung was excised and perfused through the pulmonary vasculature (arterial pressure = 15 cm H₂O) with Krebs Ringer bicarbonate solution (KBR) that contained 6% colloid (1% bovine serum albumin and 5% Ficoll). The outflow of perfusion fluid from the pulmonary vein ranged from 1.5 to 3 ml/min and was collected in a fraction collector. The lobe was filled through the main bronchus with KBR that contained radiolabeled high molecular weight dextran. After one hour, the lobe was drained and slices, 0.5 to 1 mm thick, were prepared with a Stadie Riggs tissue slicer and blotted on KBR moistened filter paper.

Table 1 summarizes the composition of slices from O₂-filled and KBR-filled lobes. The conditions of each experiment were designed to assess the effects of fluid filling, perfusion, perfusion with reduced colloid and agents that interfere with the cell's ability to regulate its internal composition. Compared with slices from excised gas-filled lungs, tissue from perfused, fluid-filled lungs contained 30% more water, a volume that corresponds closely to the volume of dextran distribution. This volume represents luminal solution that was trapped in the slice and was not affected by the magnitude of lobe filling volume over a range of 4 to 10 ml/kg body weight (1 to 4 ml/lobe). Changes in electrolyte composition of the fluid-filled lung are compatible with the addition of NaCl solution to the slice. Perfusion increases the NaCl content slightly, a change which is mostly accounted for by the replacement of blood with KBR.

When colloid in the perfusion fluid was reduced from 6 to 1%, an additional liter of fluid/kg dry weight was added to the slice. Increases in NaCl content were compatible with the addition of KBR to the lobe. This change probably reflects interstitial edema that results from the decreased osmotic driving force in the vascular compartment.

Table 1. Electrolytes and Water of Slices
from the Left Lobe of the Rat Lung

Conditions	n	H ₂ O (1/kg dry wt)	V _{dextran} ^f	Na ⁺	K ⁺	Cl ⁻
				(mg/kg dry wt)		
Mean (+SE)						
<u>O₂-Filled</u>						
Unperfused	15	4.5(+0.1) ^g	--	458(+13) ^g	339(+6)	404(+12) ^g
<u>KBR^a-Filled</u>						
Unperfused	3	5.6(+0.1)	1.2(+0.1)	600(+32)	334(+11)	505(+21) ^h
KBR perfused ^b	7	5.9(+0.1)	1.2(+0.1)	639(+17)	315(+11)	595(+14)
Low colloid ^c perfused	8	7.1(+0.3) ^h	1.4(+0.1)	783(+84) ^h	349(+14)	725(+63) ^h
Ouabain ^d perfused	3	6.1(+0.2)	1.3(+0.2)	788(+16) ^h	209(+32) ^h	617(+43)
Metabolic ^e inhibitor perfused	4	6.5(+0.4)	2.6(+0.8)	809(+14) ^h	163(+16) ^h	640(+50)

^aKrebs bicarbonate Ringer solution.

^bKrebs bicarbonate Ringer solution with 1% bovine serum albumin (BSA) and 5% Ficoll (w/v).

^cKBR with 1% Ficoll or bovine serum albumin.

^dKBR with 1% BSA, 5% Ficoll, and 10⁻³ M/l ouabain.

^eKBR with 1% BSA, 5% Ficoll, 10⁻³ M/l iodoacetamide, and 10⁻³ M/l NaCl.

^fVolume of distribution of ¹⁴C-carboxyl dextran that was added with KBR in the airspace.

^gO₂-filled value significantly different (P<.05) from values for KBR-filled unperfused.

^hKBR-filled value significantly different (P<.05) from values for KBR perfused.

The addition of ouabain to the perfusion fluid (final concentration = 10⁻³ M/l) increased slice Na⁺ and decreased K⁺. These results suggest that cells exchange K⁺ for Na⁺ without tissue swelling. Ouabain has been reported to induce K⁺-Na⁺ exchange without swelling in other tissues (19). Cation exchange was also observed when metabolic inhibitors were included in the perfusion fluid. In addition, there was a tendency for a small volume of NaCl solution to be added to the slice. The increased dextran volume of distribution may reflect increased permeability of membranes of cells of the epithelial barrier. However, the permeability coefficient that was calculated from

the rate of dextran appearance in the venous outflow was usually below 10^{-9} cm/sec, a value lower than the coefficient for albumin flow across perfused, fluid-filled dog lung (15).

The usefulness of the slice protocol is illustrated by the experiment shown in Table 2. The addition of amphotericin B to the fluid in the airspace of the perfused lobe nearly doubled the concentration of K^+ that was recovered in the fluid after one hour (38). Since water and electrolytes and the volume of recovered fluid did not change, the increase in luminal K^+ cannot be attributed to a loss of tissue K^+ . The electrical potential difference between the airspace and perfusion fluid seldom exceeded 6 mV (lumen negative) so that the transepithelial K gradient does not reflect a passive distribution. We conclude that amphotericin induces the active secretion of K^+ into the lumen, an effect which has been reported for epithelia of colon (4), frog skin (21), toad urinary bladder (9), and bullfrog lung (11). Further, the increase in K^+ in the lumen of the rat lung could be reduced by adding ouabain to the perfusion fluid, suggesting the participation of Na^+-K^+ ATPase in K^+ transport.

Excised Rat Trachea In Vitro

The contribution of the airway epithelium to the respiratory lining was estimated from functions of the excised rat trachea (31). To make use of nearly all of the small tracheal surface the entire cylinder was excised and tied to the arms of an inverted "y"-tube. The tracheal cylinder was suspended horizontally in an outer bath of KBR at 37°C. Bubbles of 5% CO_2 -95% O_2 and a gas lift in the tubing recirculated KBR through the lumen of the trachea. Transtracheal electric p.d. was monitored between KBR-agar bridges near the midpoint of the tracheal cylinder and in the outer bathing solution. The bridges were connected through calomel cells to a high impedance voltmeter. Current from a d.c. voltage source was passed between an axial silver-silver chloride wire in the lumen and an outer silver-silver chloride foil that surrounded most of the trachea. Cysteine (1 mM/l) was included in the bathing solutions to complex any silver that might have been released from the electrodes. Unidirectional fluxes of radioactive solutes were determined by adding the tracer to one bathing solution and determining the rate of radiolabel appearance in the other bath.

Under open circuit conditions 14 tracheas exhibited a transmural electric p.d. of $9.3 (\pm 1.2 \text{ SE})$ mV, lumen (mucosa) negative, and a d.c. conductance of $11.0 (\pm 1.2)$ mS/cm². These bioelectric properties remained constant for at least two hours. Unidirectional ²²Na fluxes across the short-circuited trachea (transmural voltage clamped at zero) were asymmetric and indicative of an active reabsorption (net mucosal to serosal flow) of $1.7 \mu\text{eq Na}^+/\text{cm}^2, \text{ hr}$. Under the same conditions, ³⁶Cl fluxes revealed an active secretion into the

Table 2. Effect of Amphotericin B on the Composition of Perfused Lung and the Fluid Added to the Airspace

Airspace fluid	Tissue			Recovered airspace fluid	
	n	H_2O (1/kg dry wt)	Na ⁺ K ⁺ Cl ⁻ (meq/kg dry wt)	Volume	K ⁺ added
			Mean ($\pm$ SE)		
KBR	6	5.7($\pm$ 0.1)	59.3($\pm$ 11) 350($\pm$ 6) 559($\pm$ 10)	82($\pm$ 6)	117($\pm$ 4)
KBR + 10^{-5} M/l amphotericin	3	5.7($\pm$ 0.1)	657($\pm$ 14) 369($\pm$ 19) 580($\pm$ 10)	71($\pm$ 5)	179($\pm$ 5) ^a

^aSignificantly greater ($P < .05$) than the value for KBR without amphotericin.

lumen of $1.9 \mu\text{eq}/\text{cm}^2$, hr. Furthermore, the sum of the net Na^+ and Cl^- movements accounted for 95% of the current required for short-circuiting. Cl^- secretion and Na^+ reabsorption have been reported for excised, short-circuited dog trachea (26). ^{42}K and ^{14}C mannitol unidirectional fluxes were symmetric and, therefore, consistent with passive transfer.

Figure 1 depicts the relationship between the passive flow of each solute and conductance. The linear relationship between mannitol and conductance and the intercept near zero suggest that most of the current carrying electrolytes and mannitol traverse the same path. A comparison of points on the least square lines at any common conductance describes the relationship between the permeability coefficients (P) for the solutes. The ratio of P_{Na^+} or P_{K^+} to P_{mannitol} approximates the ratio of the free diffusion coefficients for each solute pair (see Table 4). These results are compatible with an equivalent pore radius in excess of 4 nm. This estimate and the implication that mannitol, a solute restricted to the extracellular compartment, and the electrolytes are moving through the same channel suggest that this path is paracellular. Cl^- movement relative to mannitol flow was less than the free diffusion ratio and might be explained if the paracellular path is lined with fixed negative charges.

The addition of amphotericin to the luminal bathing solution (final concentration = 10^{-6} M/l) increased unidirectional fluxes for all solutes from two- to fivefold. Specifically, net secretion of K^+ could not be detected.

Determination of Alveolar Epithelial Na^+ Permeability and K^+ Secretion

If the trachea is accepted as a model for the remainder of the airways, then permeability and electrolyte transport by the gas exchange epithelium can be deduced from concordant measurements in trachea and the perfused, fluid-filled lobe (Table 3). The unidirectional flow of Na^+ into the lumen of trachea exceeded flow in the same direction in the lobe by two orders of magnitude. Since the lobe is lined by both alveolar and airway epithelia with a surface area ratio of about 700 to 1, the passive Na^+ permeability of the alveolar epithelium should be slightly less than that of the lobe. By the same reasoning the increase in Na^+ permeability induced by luminal amphotericin was considerably smaller in the lobe than in trachea, suggesting an even smaller increase in the alveolar epithelium.

K^+ secretion into the lumen of the lobe was induced by amphotericin but could not be detected in trachea. This does not exclude the possibility that K^+ secretion of the magnitude measured in lobe might be present in trachea.

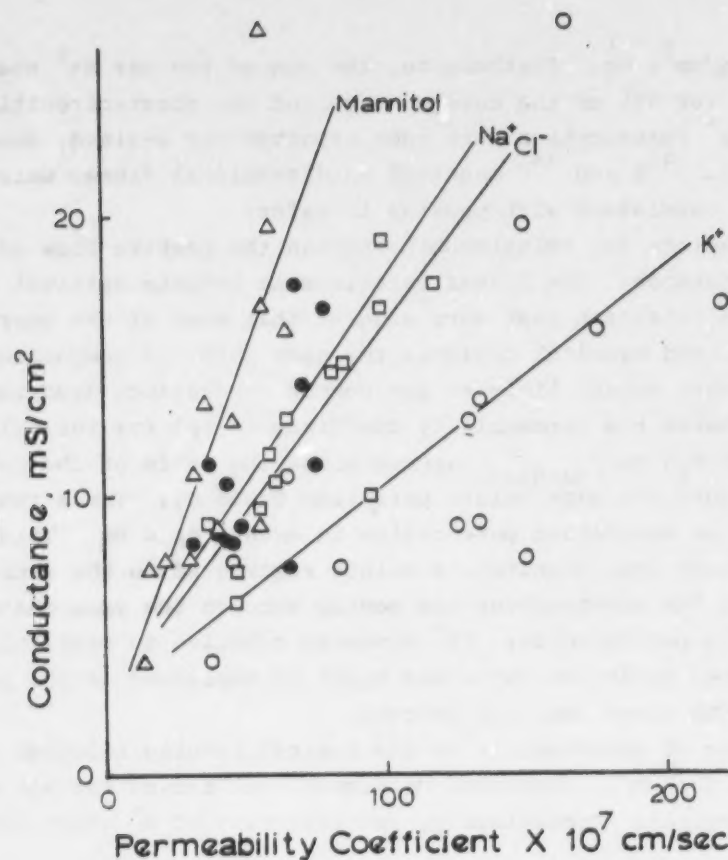


Figure 1. The relationship between d.c. conductance and steady-state, unidirectional solute flow across excised rat trachea in vitro. Permeability coefficients were calculated from the unidirectional passive flux of tracer (P = radiolabel flux per unit area/radiolabel concentration in the source). Each symbol [$\circ$ - K^+ , $\bullet$ - Na^+ (serosal to mucosa), $\square$ - Cl^- (mucosa to serosa), and Δ -mannitol] represents data from one trachea. Lines are fitted to the points by the method of least squares.

However, it is clear that a K^+ secretion of about $50 \mu\text{eq}/\text{cm}^2$, hr would have to be induced by amphotericin in the airways of the lobe to account for entire change in luminal K^+ concentration. Since this secretion could be easily measured in the trachea it is reasonable to conclude that most of the K^+ is secreted by the alveolar epithelium.

Excised Bullfrog Lung

An alternative approach to the characterization of alveolar epithelial permeability and ion transport is to exploit species with large areas of

Table 3. Cation Flow Across Excised Rat Trachea and the Perfused Left Lobe of the Rat Lung

Flow	Lobe ^a	Trachea
	(μeq/cm ² , hr)	
Na ⁺ (passive)		
Untreated	0.029 ^b	2.7 ^c
Amphotericin B	0.042 ^b	15.5 ^c
K ⁺ (net secretion)		
Untreated	0.0013 ^d	<0.05 ^e
Amphotericin B	0.0090 ^d	<0.05 ^e

^aEstimated surface area = 1400 cm².

^bCalculated from the rate of change in the amount of Na⁺ in airspace fluid, a KBR with Na⁺ replaced by choline.

^cSerosal to mucosal flux.

^dThe rate of change in the amount of K⁺ in the KBR in the airspace.

^eSerosal to mucosal flux - mucosal to serosal flux.

intact alveolar surface that can be separated easily from the airways. Each lobe of the lungs of frogs and toads is a single, large alveolus more than a centimeter in diameter that is connected by a short airway to the trachea (23). The luminal surface of the lobe is lined by a continuous epithelial monolayer. Most of this layer is comprised of type I and type II pneumocytes (20). A few ciliated cells cover the tips of a fibrous trabecular network in the interstitium which also contains blood vessels, axons, and muscle bundles. The interstitium separates the epithelium from a continuous pleural covering.

The alveolar sac can be opened and mounted as a planar sheet between Ussing chambers. With identical Ringer solutions bathing both sides of the lung, a transmural bioelectric p.d. of nearly 20 mV (lumen negative) and d.c. conductance of 1.4 mS/cm² were measured (5). Direct and indirect studies indicate that the epithelium is the site of the biopotential and the major resistance to transmural ion flow (5, 8).

The passive permeability of the bullfrog lung to electrolytes is similar to that reported for the fetal and fluid-filled adult mammalian lung (7). In addition, the bullfrog lung, like the fetal lung, actively secretes Cl⁻ and other halides into the lumen. Cl⁻ transport equals the short-circuit current and can be inhibited by metabolic inhibitors or Br⁻.

Studies that were designed to establish the dimension(s) of an equivalent pore(s) have been equivocal. Table 4 summarizes the permeability co-

Table 4. Solute Permeability of Excised Bullfrog Lung

Solute	Radius (μ m)	n	$P \times 10^7$ ^a (cm/sec)	$P_{\text{solute}}/P_{\text{mannitol}}$ mean (+SE)	$D_{\text{solute}}/D_{\text{mannitol}}$ ^b
³⁶ Cl	0.19	5	15.2(+2.6)	6.9(+0.7)	2.87
²² Na	0.29	5	9.5(+0.8)	5.4(+1.0)	1.87
¹⁴ C-mannitol	0.4	29	2.7(+0.2)	1	1
⁵⁷ Co-B ₁₂ ^c	7.2	3	0.08(+0.03)	0.05(+0.01)	0.50
³ H-methoxy inulin (unfiltered)	14	3	1.2(+0.1)	0.72	0.32
³ H-methoxy ^d inulin (gel filtered)	14	2	0.40	0.12	0.32
¹⁴ C-methoxy ^d inulin (gel filtered)	14	2	0.25	0.06	0.32

^a Permeability coefficient for the unidirectional flux of tracer from the luminal to pleural bathing solution.

^b Ratio of the free diffusion coefficients.

^c Cyanocobalamin.

^d Chromatographed on a Sephadex G-100 column. About 25% of the total radioactivity was selected from the peak and an equal number of fractions on either side of the peak.

efficients for the passive flux of solutes of different size across the short-circuited lung. In contrast to the canine trachea, the coefficients for Na^+ and Cl^- movements relative to that of mannitol are greater than the free diffusion ratio. These results suggest that mannitol movement and the flow of the larger cyanocobalamin and inulin molecules are restricted, i.e., the apparent area for large molecule diffusion through aqueous channels is less than that for small molecules. However, the permeability coefficient measured for inulin depends on the "purity" of the tracer species. Commercial tritiated methoxy-inulin contains small fragments which permeate the lung rapidly, resulting in a ratio of inulin to mannitol flows which is more than twice the ratio predicted for free diffusion. Partial purification of inulin by fractionation on polyacrylamide gel results in a tracer species that is restricted, but the possibility of minor contamination by small fragments cannot be excluded. The contribution of this contamination to the apparent inulin flux is magnified when restriction is severe. This uncertainty leads to the calculation of several pore radii for the lung (Fig. 2). Whereas the flows of Na^+ , Cl^- , and cyanocobalamin relative to that of mannitol describe a pore radius of 1 to 2 nm, the most slowly permeating inulin species appears to require a radius of 3.5 nm. Despite these reservations, most of the data favor the notion that the passive flow of hydrophilic molecules across the bullfrog lung involves pores that resemble those of fetal or adult mammalian lung rather than the transmural channels in the tracheal mucosa.

The potential value of pore size estimates can be illustrated by experiments that assess the effects of pH on bullfrog lung permeability. Exposure of the mucosal (luminal) or serosal (pleural) surface of the lung to unbuffered Ringer solutions that contain HNO_3 , H_2SO_4 , or HCl induce identical effects. Bioelectric properties do not change until the pH of the bathing solution falls below three. Then conductance increases several fold. Volume flow in response to an osmotic gradient rises. Table 5 illustrates the effects of exposing either surface of the lung to HCl . The relatively low pH of the other bathing solution reflects the permeation of protons through the lung during the three-hour experiments. In both experiments an increase in conductance was paralleled by a dramatic increase in the mannitol and Cl^- permeability coefficients. The ratio of the coefficients ($P_{\text{Cl}^-}/P_{\text{mannitol}}$) approached the value for diffusion in free solution. This fall can be explained by the expansion of existing channels to a radius greater than 4 nm and/or the creation of new large pores.

Whereas the effects of mineral acids on lung permeability are not subtle, a number of chemical agents induce selective changes in functions of the bullfrog alveolar epithelium. The replacement of NaCl in the mucosal bathing solution by NaNO_3 inhibits short-circuit current by 40% but does not

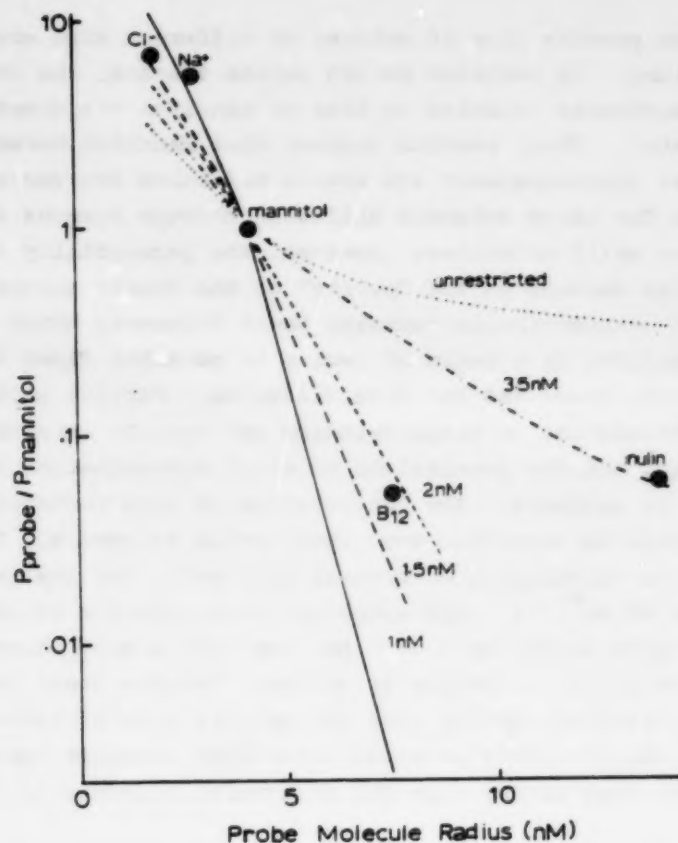


Figure 2. Estimation of equivalent pore radius from the permeability coefficients for solute flow. The relationship between the permeability coefficient (P) and pore radius is given by:

$$P_{(\text{cm/sec})} = \frac{D}{\Delta x} F(a/r) \quad (28)$$

where D = the diffusivity in aqueous solution (cm^2/sec); Δx = the path length for diffusion (cm); $F(a/r)$ = the function that describes steric and frictional interactions between solute and pore, specifically $[1 - (a/r)^2]^{1-2.1} [(a/r) + 2.09(a/r)^3 - 0.95(a/r)^5]$ in which a = solute radius and r = pore radius. By dividing the permeability coefficients for other probe molecules by the coefficient for a reference solute, mannitol, Δx is eliminated:

$$\frac{P_{\text{probe}}}{P_{\text{mannitol}}} = \frac{D_{\text{probe}}}{D_{\text{mannitol}}} \frac{F(a_{\text{probe}}/r)}{F(a_{\text{mannitol}}/r)}$$

The lines join predicted ratios of the permeability coefficients for each solute-mannitol pair. The ratios were calculated from assumed pore radii and literature values for a and D.

Table 5. Effect of Bathing Solution pH on the Solute Permeability of Excised Bullfrog Lung

Luminal pH	Pleural pH	Solute	n	G^a (mS/cm ²)	$P \times 10^7^b$ (cm/sec)	$P_{Cl^-} / P_{mannitol}$		$D_{Cl^-} / D_{mannitol}$	
8.3	8.3	³⁶ Cl ⁻	5	0.9	15.2 ^c	6.9		2.9	
		¹⁴ C-mannitol				1		1	
2.5	6.3	³⁶ Cl ⁻	2	6.7	669 ^c	2.9		2.9	
		¹⁴ C-mannitol				1		1	
6.3	2.5	³⁶ Cl ⁻	2	7.4	614 ^d	3.4		2.9	
		¹⁴ C-mannitol				1		1	

^ad.c. conductance.

^bpermeability coefficient for the contemporaneous flux of radio-mannitol and Cl⁻ across the same lung lobe.

^clumen to pleural flux.

^dpleural to lumen flux.

affect conductance. Bioelectric properties are not affected by serosal exposure to NaNO_3 (8). The mode of NO_3^- action is not known, but unpublished flux experiments demonstrate that Cl^- flow toward the lumen and the transmural movement of Na^+ in either direction are unaffected.

Amphotericin B in the mucosal solution induces not only the secretion of K^+ into the lumen but also a paracellular K^+ selective pathway (11), an effect which has also been described for toad urinary bladder (7).

High concentrations of HgCl_2 in the mucosal solution (10^{-5} to 10^{-4} M/l) lead to an increase in conductance and general ion permeability and inhibition of active Cl^- secretion (8). However, the flow of water in response to a transmural osmotic gradient is not affected (12). When exposure to Hg is followed after one minute by the addition of an excess of a sulfhydryl agent, such as dimercaprol, there is a sustained 40% increase in short-circuit current and in the secretion of Cl^- , but neither conductance nor unidirectional fluxes of Na are affected significantly (12).

Finally, the beating of cilia on the cells that cover the trabecula is inhibited by the addition of CdCl_2 to the luminal solution (concentration range = 10^{-5} - 10^{-3} M/l) (10). The metal also decreases the rate of tissue oxygen consumption but does not affect bioelectric properties or transmural ion movement. Studies with alveolar epithelial cells that were disaggregated from the lung surface by perfusion through the pulmonary vasculature with collagenase show that Cd complexed with bovine serum albumin or hemoglobin does not alter ciliary motility (13). However, these extracellular proteins do not reverse the effects of Cd that has already reacted with the cells. In contrast, Cd exposed cells washed with permeant sulfhydryl agents, such as mercaptoethanol, dimercaprol, or cysteamine, partially or completely recover oxygen consumption and ciliary motility (14). These observations suggest that the inhibition of ciliary motility requires the interaction of Cd with intracellular ligands. Measurements of the Cd binding which persists after the sulfhydryl agent wash suggest that no more than 30% of the total Cd ligands participate in the inhibition of ciliary motility.

CONCLUSIONS

Evidence from fluid-filled, perfused rat lung lobes, rat trachea, and bullfrog lung indicates that the alveolar epithelium restricts the movement of small hydrophilic molecules. The large epithelial surface of the alveolar epithelium is shunted by the airway epithelium, a less restrictive barrier. Chemical agents, such as amphotericin B, can induce quantitatively and, perhaps, qualitatively different effects in the gas conducting and exchanging epithelia. Whereas exposure to acids ($\text{pH} < 3$) induces a general increase in solute and water permeability and in pore radius, other edemagenic agents,

such as HgCl_2 and CdCl_2 , affect ion secretion and ciliary motility without altering the permeability of the alveolar epithelium.

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DISCUSSION

DR. DAVIS: I have one technical question relating to the work on rat trachea and your system for measuring short circuit current. As you know, the original Ussing technique depends on the presence of a uniform electrical field across the tissue. Your approach may have been described before. I thought about how you measure short circuit current; it's always worried me that you can't meet those conditions by having a wire down the middle or something around the outside. Could you say something about that?

DR. GATZY: Certainly. The requirement is that the field should be uniform. This will result when an axial wire is placed in the center of the tracheal cylinder and current is passed between this wire and an outer cylindrical foil. As long as the wire and foil are of uniform composition and plated uniformly, you ought to get a uniform current. That's what we tried to do. This is not the first time that this technique has been used. Clarkson and Toole used this approach with the intestine. The intestine was mounted vertically and the rather long piece of intestine required large silver electrodes. So the technique has been used before with some success. There may be a problem in positioning the electrode inside the very small lumen of the trachea.

DR. DAVIS: That may, in fact, be a part of the problem with your residual conductance.

DR. GATZY: We have thought of that and have attempted to look into the question. By moving the electrodes around to the geometric extremes, it looks like the maximum error is of the order of 15 percent, but when you add this change in driving force to flow in both directions, it's a possibility.

DR. DAVIS: It's encouraging that you got matching results. You know that John Mangos has done similar kind of work using rat trachea. That's published in the Cystic Fibrosis Abstracts.

DR. GATZY: There is a problem with measurements of net flow as defined by dilution of inulin in the lumen because inulin permeates through the tissue. Those results must be scrutinized carefully to determine whether there really is net addition of volume to the lumen.

Under short-circuit conditions we observed sodium reabsorption and chloride secretion, much like the flows across the dog trachea except that these flows seem to be more evenly balanced. As you know, opening the circuit tends to counter the flows measured under short-circuit conditions. I would predict little or no net ion flow across rat trachea under open circuit conditions.

DR. BELL: How was albumin on the alveolar surface determined?

DR. GATZY: As I understand Reifenrath and Zimmerman's work, protein that was recovered in fluid from the alveolar surface was not correlated with the volume of collected fluid and was considered to result from "contamination." So, under the normal resting situation, at least in the rat, they consider protein to be absent from the alveolar fluid. How it gets there after treatment with the edemagenic agents is still an open question. Protein could leak through the junctions of the alveolar epithelium, or through the airway epithelium and then run down into the alveoli. There seems to be no evidence to clearly distinguish between these two possibilities.

DR. BELL: Do you believe that under normal conditions in the lung you would not find a protein like albumin on the alveolar surface?

DR. GATZY: The only study that sampled alveolar surface fluid directly dismisses this possibility.

Session II:
Respiratory Tract and Host Defense

Neutral Proteases of Human Polymorphonuclear Granulocytes: Putative Mediators of Pulmonary Damage

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Neutrophil polymorphonuclear (PMN) granulocytes and their neutral elastases are among today's leading candidates for principal mediators of chronic obstructive pulmonary disease due to panacinar emphysema (9). The concept of these cells and their enzymes as mediators of lung damage fits well with the established anatomical and functional importance of elastic fibers in alveolar septa and respiratory bronchioles. Figure 1 shows the respiratory bronchiole leading into the pulmonary alveoli. Figure 2 shows the numerous elastic fibers woven through the alveolar and bronchiolar walls. This concept also fits with reports that in emphysematous lung elastic fibers have been damaged. Elastase itself affords an element of specificity that enhances the chance that it is important in this picture of damage since purified collagenases and trypsin seem unable to induce experimental emphysema. As things stand, the most successful animal models of human emphysema depend on intratracheal installation of an elastolytic protease such as papain or elastase from polymorphs. Intravenous injections will work but require much higher doses of enzyme. In hamsters 0.2-0.5 mg/100 g of elastase by intratracheal installation initiates irreversible lung disease. The exact role of the granulocytes, other than their service as ready sources and vehicles for elastase, is still uncertain. However, there appear to be two phases for their participation in lung damage. There is recruitment of the cells and there is exocytosis (degranulation) of the elastase. Recruitment is likely to depend on deposit in respiratory

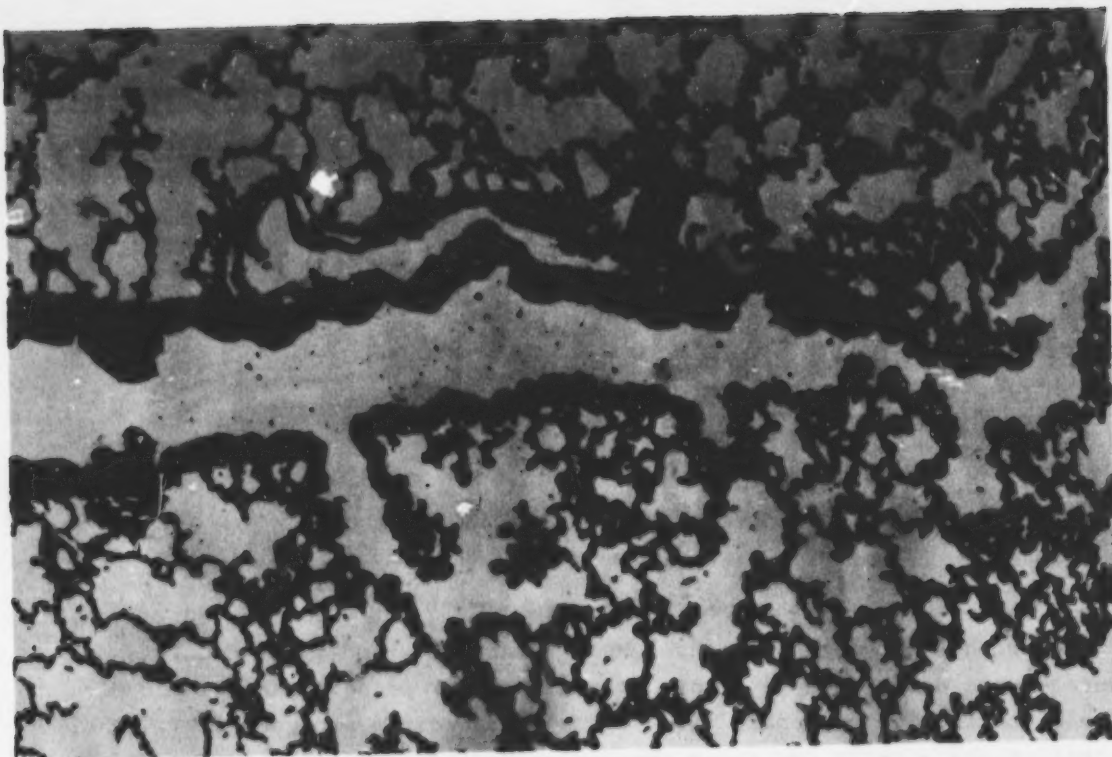


Figure 1. Normal bronchiole. H and E stained histological section. Note respiratory bronchiole (left) and alveoli. Courtesy of Dr. E. MacRae, Department of Anatomy, University of North Carolina.

bronchioles and alveoli of foreign particulate matter with cytotoxic materials absorbed onto their surfaces (10). Exocytosis of elastase and access of the enzyme to elastin fibers may depend on the direct effects of cytotoxic chemicals that damage the PMN (2) and the counter-inhibitory effects of these chemicals acting on protease inhibitors from plasma (8). Exocytosis of proteases may also be induced with chemicals absorbed to particles phagocytized by the polymorphs.

In view of the rather limited knowledge of the circumstances of local involvement of PMN in the lung, I have chosen not to discuss them but to discuss some pertinent aspects of the cell biology of PMN neutral proteases about which many details are available. I shall try to relate these in a speculative way to the events that may be taking place in the lung. In particular, I shall discuss the kinds of proteases associated with PMN, the way in which they are packaged, the ways in which they come to be released from PMN, and some aspects of their action on connective tissue.

Human PMN have three neutral proteases that have been studied in

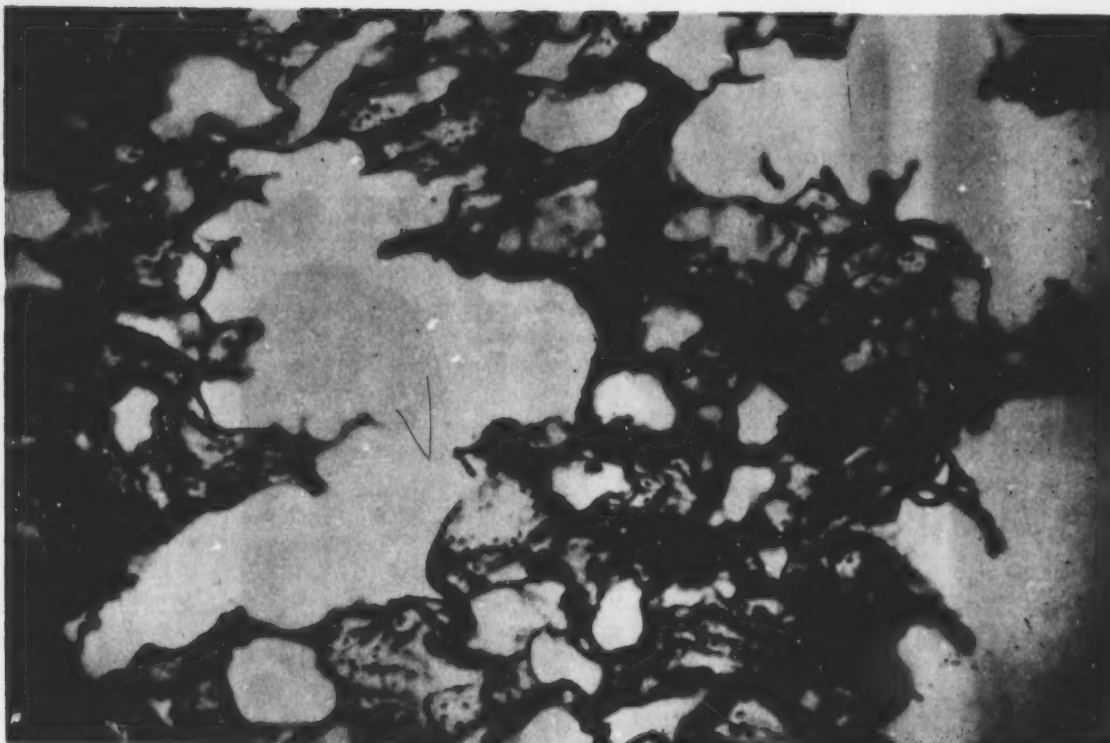


Figure 2. Lung. Orcein (elastin) stain. Note elastin fibers about alveoli. Dots are nuclei. Courtesy of Dr. E. MacRae.

considerable detail, an elastolytic enzyme, a chymotrypsin-like cathepsin G, and a collagenase (1, 3, 11). There are, in addition, much less extensively studied enzymes--a plasminogen activator (4) and proteinase 3 (P_3) that has hardly been characterized at all (1). The elastase and the cathepsin G are serine esterases active against a variety of large molecules such as casein, but the important thing about elastase is its unique capacity to cleave the helical peptide of elastin. Being serine esterases, these enzymes are irreversibly inhibited by chloromethyl ketone derivatives of synthetic peptides and by sulfonyl fluorides. The elastase is highly reactive with peptide derivatives that have alanyl-alanyl-prolyl-valyl sequences. Cathepsin G is highly reactive with peptides containing phenylalanine (11). The enzymes are readily demonstrated with polyacrylamide gel electrophoresis. They are also inhibited by naturally occurring protease inhibitors found in plasma, α_1 -antitrypsin and α_2 -macroglobulin and α_1 -antichymotrypsin (6). The collagenase is not an esterase, it is unaffected by the chloromethyl ketone inhibitors. Since it is a metallo-enzyme it is inhibited by metal chelators such as ethylenediaminetetraacetic

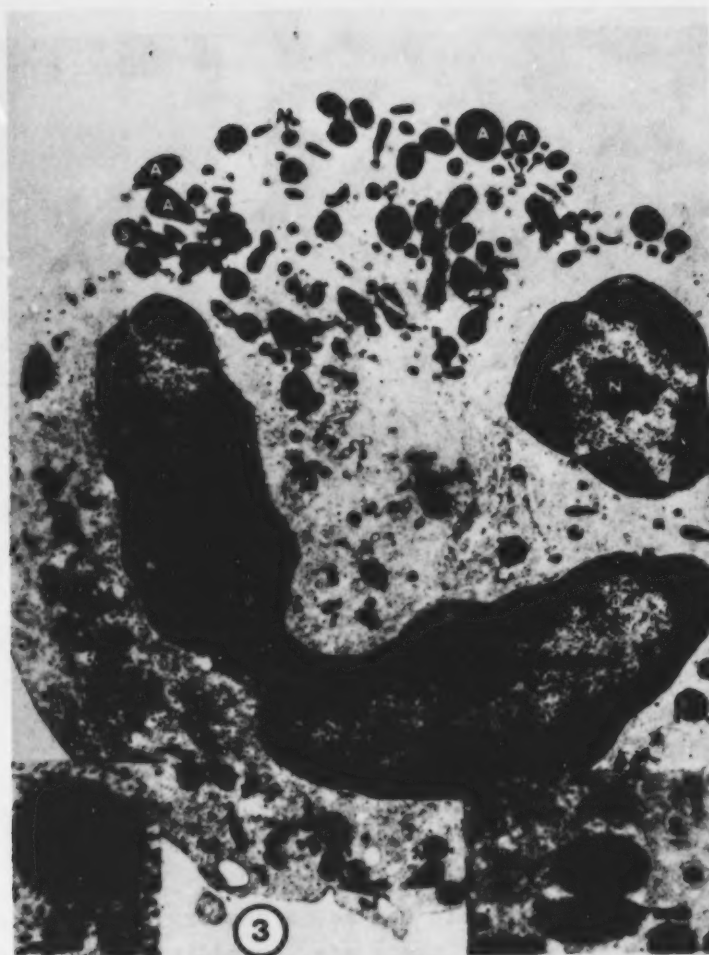


Figure 3. Human, normal, resting neutrophil. A, azurophil granules; S, specific granules; M, vestigial mitochondria; G, Golgi apparatus; C, centriole. Fixation 0.5% glutaraldehyde, postfixed in osmium tetroxide. Stained with uranyl and lead. Insets: left, an enlarged detail of figure; right, granules stained only with peroxidase stain and uranyl. X30,000.

acid (1). The synthetic inhibitors are important to studies on the catalytic action of the serine esterases. Moreover, some thought has been given to the possible therapeutic use of the chloromethyl ketone inhibitors to inhibit elastase (12). More will be said below about plasma inhibitors.

Returning to the cell biology of the neutral proteases, it is important to know they are carried inside the membranes of granules in the PMN cytoplasm. Figure 3 shows a transmission electron microscope view of the

granule apparatus of a resting human PMN. Note that the granules appear heterogeneous, that is, there are many different sizes and electron densities. In spite of the confusing varieties, there are only two biochemically distinct kinds, the specifics or secondary granules and the azurophils or primary granules. Both granule types are formed while polymorphs are still in the marrow. Azurophils are formed in the promyelocytes and specifics are formed in the myelocytes (13). The azurophils tend to be large, about 0.3 micrometers in diameter. The specifics tend to be smaller, about 0.1 micrometers in diameter (15). The important point is that the elastase and cathepsin G are unequivocally carried in azurophil granules (1, 3). Collagenase, however, is in the specifics (1 and observations, this laboratory). We know this as a result of cell fractionation experiments. Thus purified PMN can be broken up in a homogenizer, their granules released, and the nuclei containing the anti-proteases removed. When this mixture of granules is centrifuged through a sucrose density gradient, several bands containing different subcellular particles form in the gradient. Figure 4 shows a 60 ml centrifuge containing such a sucrose density gradient. The subcellular particles have been centrifuged to density equilibrium. The specific granules are seen clearly as band II_f. The azurophil granules are seen as two bands marked III_s and III_f (15). Pumped out of the centrifuge tubes and analyzed, these bands of granules are associated uniquely with certain constituents. Table 1 summarizes a few of the constituents of the major granules. The azurophils contain elastase, cathepsin G and myeloperoxidase (MPO). They also contain acid hydrolases--phosphatase, β -glucuronidase, and lysozyme. The MPO, besides being strongly antibacterial, serves as a unique marker for azurophils (15). Most of these enzymes are very cationic proteins. The ones in the table are ordered according to their degree of cationicity. The cationic property may promote their binding to connective tissues. The specific granules have lysozyme and, in addition, they have apo-lactoferrin, an 80,000 dalton, iron binding, intracellular analogue of transferrin. It forms a specific marker to trace the fate of these granules (15). Specific granules also contain a collagenase that is latent as we shall see. Figure 5 shows the results of biochemical and immunochemical analysis of the fractions. The relative distribution of the enzymes is shown in relation to the gradient in which the particles are depicted as moving from left to right. Enzyme peaks are to be compared with the positions of the various granule populations marked by daggers and by the MPO in the azurophils and the lactoferrin in the specifics. The elastase is distributed exactly as are the azurophil granules. Not shown here but with exactly the same distribution in the azurophil granules is cathepsin G (11). From this we conclude that both elastase and cathepsin G are azurophil granule enzymes.

It can also be seen that the collagenase is in the specific granules.

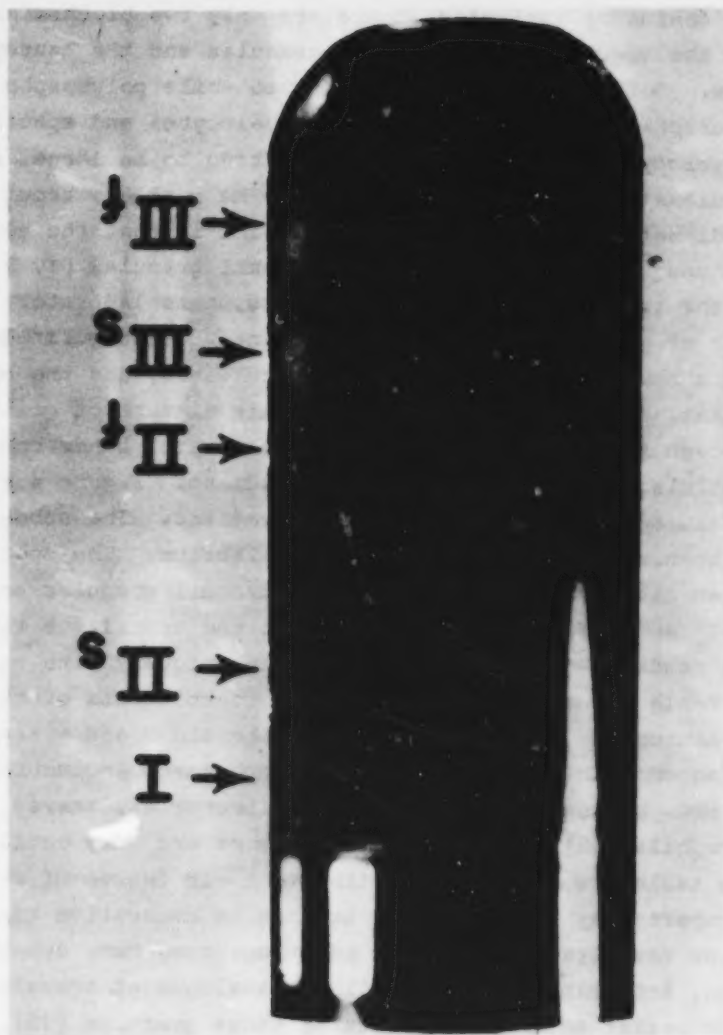


Figure 4. Human neutrophil polymorphonuclear granulocyte granules separated on sucrose density gradient. The II_f band comprises the specific granules. III_s and III_f comprise two subclasses of azurophil granules. I, cell membranes; II_s mitochondria.

Curiously, the collagenase is doubly latent, that is, it cannot be detected in intact granules. Moreover, it cannot be detected when the specific granule membranes have been removed unless it is activated by a proteolytic enzyme. There is an expected latency in all granule enzymes. It is due to the membranes that surround the granule enzymes, rendering them inaccessible to substrate until they are treated to remove the membrane barrier as was

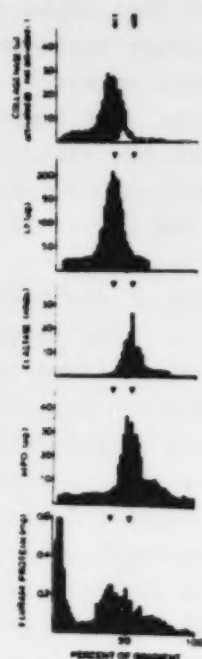


Figure 5. Histogram showing activities of collagenase (white histogram latent, black histogram collagenase with trypsin activation), lactoferrin (specific granule marker), myeloperoxidase (azurophil granule marker), elastase, and amounts of protein from sucrose density gradient similar to Figure 4.

Table 1. Some Constituents of the Major Granules of Neutrophil Polymorphonuclear Granulocytes

Azurophil (primary) granules	Specific (secondary) granules
Acid Phosphatase	Lysozyme
Acid β Glucuronidase	Lactoferrin
Myeloperoxidase	Collagenolytic Enzyme
Elastase	
Lysozyme	
Cathepsin G	

done here to measure the MPO, elastase, and cathepsin G. Latency could not be eliminated so simply with the collagenase. The small white histogram shows the small activity detected with only membrane removal. The larger black histogram shows the activity and distribution of collagenase with

membrane removed and latent enzyme activated by another protease. Trypsin was the first protease we found that could activate the collagenase.

The most effective collagenase activator turned out to be the highly purified cathepsin G of azurophil granules. Elastase of azurophil granules was less effective than cathepsin G or trypsin. This was shown with the digestion of salt soluble guinea pig skin collagen and was measured with release from the collagen of biologically incorporated ^{14}C -labeled glycine and proline. The most active enzyme was cathepsin G. Trypsin activity was intermediate and elastase was least active.

Now, why so much discussion of PMN collagenase if elastase and elastin are the most important factors in lung damage leading to emphysema? Actually there is also evidence that lung collagen is damaged as well, especially in experimental emphysema (8). Hence a mediator of that damage should be sought. For that reason I shall compare effects of collagenase and elastase on collagen and discuss further the relationships between latency of collagenase and the fate of cathepsin G, its activator. Figure 6 is from sodium dodecyl sulfate polyacrylamide gel analysis of digestion fragments cleaved from collagen by highly purified samples of these enzymes. Collagenase cleaves the helical peptides and gives rise to the characteristic $3/4$ and $1/4$ length fragments seen in gels 3, 4, 5, and 6. The presence of other cleavage products suggests that other enzymes capable of cleaving the peptides are present as well. The amount of fragments increases with time of digestion. The other gels are from collagen and enzyme controls. The cathepsin G activator is needed to activate the collagenase. Some reports suggest that, unlike elastase, cathepsin G is not secreted from PMN (14). If this is so, the collagenase may be relatively unimportant. Could elastase cleave collagen? It has been believed unable to attack native collagen, but Figure 7 shows that purified elastase monomerizes native collagen in the presence of PMN collagenase. It could do this by cleaving the N terminal peptides that connect the helical peptides into dimers, the β chains, and trimers, the γ chains. Here it is seen that with increasing time of digestion the polymers disappear, no small fragments are seen but only α chain monomers remain. Quantitatively it also seems that the α chains eventually, with time, are cleaved to very small peptides as seen in the last gel. The other gels are collagen and enzyme controls. These observations are in agreement with Starkey *et al.* (16). The relative contributions of the two enzymes in our system remain to be defined.

The crucial question, of course, is how these proteinases happen to emerge from the PMN and become available to attach to elastic and collagen fibers as purified elastase has been made to do in animal models (9). Do PMN die and autolyze leaking enzyme over the connective tissue? Evidently that is unnecessary. Phagocytosis of particles as small as 1 micrometer in

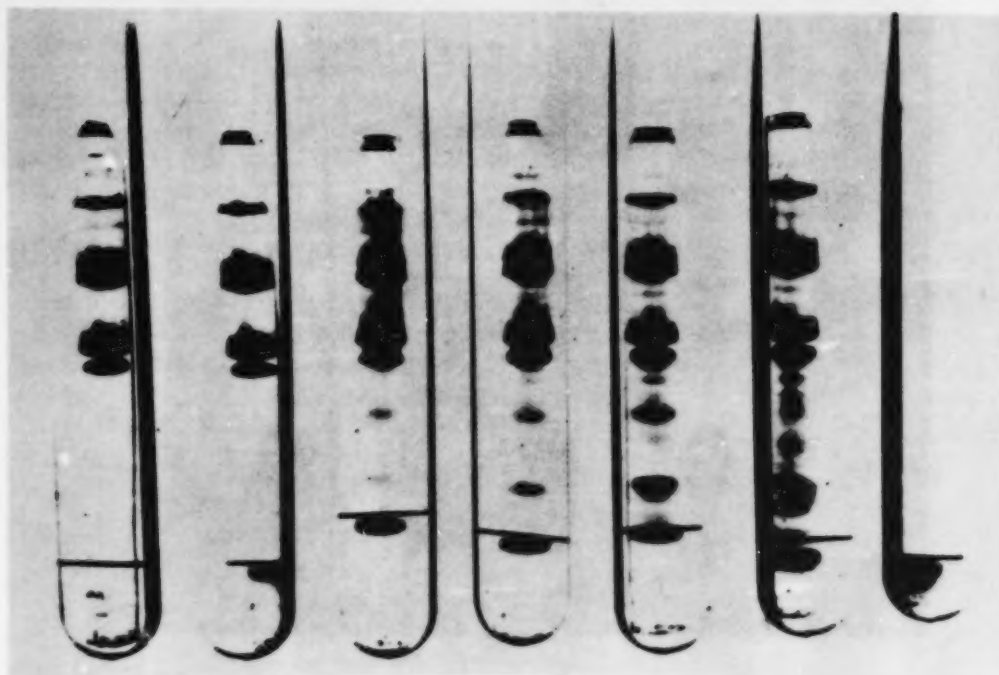


Figure 6. Cleavage products of guinea pig skin salt soluble collagen reacted with specific granule collagenase. The collagenase in this instance was activated by cathepsin G which does not attack native collagen. The cathepsin G was highly purified. The collagenase was used as specific granule extract. Far left, collagen control; second from left, collagen plus granule activated granule protein 0 time; gels 3, 4, 5, 6 - 0.5, 1, 2, 4 hours, respectively, with activated enzyme; far right, gel granule enzyme control. Note progressive appearance of α_A (3/4) and α_B (1/4) fragments. Other cleavage products not identified appeared with progression of time.

diameter, given appropriate coatings of immunoglobulins, for example, can cause secretion of elastase from human PMN. Phagocytosis of bacteria, yeast cell walls, and other particles can do this as well. This leakage is further aggravated by attempts of PMN to phagocytize surfaces they cannot surround with membrane. Henson has shown this for other PMN granule enzymes and named it frustrated phagocytosis (7).

We have been especially interested in how (and also why) PMN secrete granule enzymes during phagocytosis. It seems curious that cells so important in anti-infectious immunity as PMN should have survived with such untidy eating habits. Figure 8 is from an immunocytochemical study on

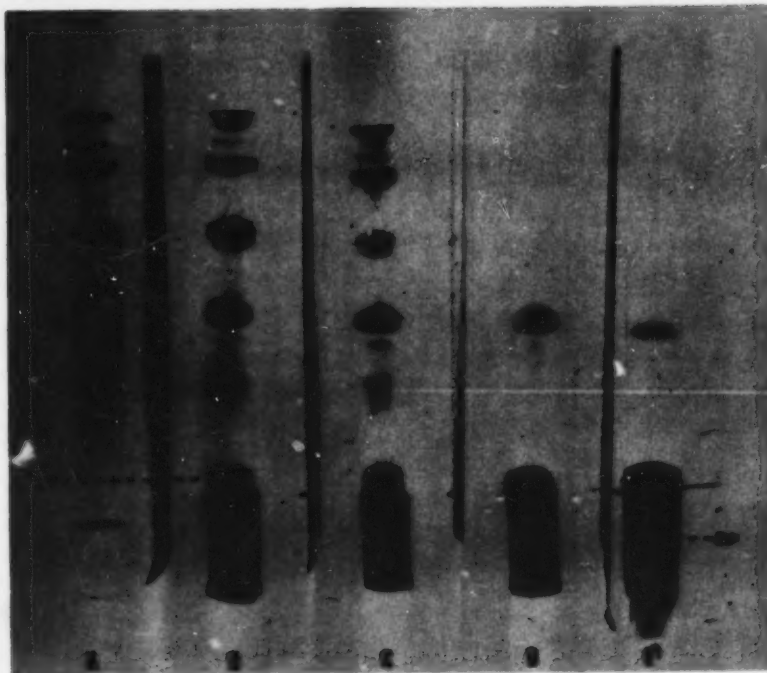


Figure 7. Cleavage fragments of collagen digested with azurophil granule elastase. Elastase cleavage of salt soluble guinea pig skin collagen. Control collagen on left (A). B, elastase (gp) plus collagen at time 0. C, D and E, time course of digestion 0.5, 1 and 2 hours incubation. Note disappearance of all but α chains.

phagocytosis of *Escherichia coli* with human PMN. The PMN were allowed to phagocytize *E. coli*, washed in saline, and fixed in 1% paraformaldehyde. The paraformaldehyde fixative leaves the cell membranes impermeable by antibody. The fixed cells were reacted with antibody against myeloperoxidase conjugated to fluorescein. Any fluorescein bound to the cells indicates antibody specifically bound to myeloperoxidase that has emerged onto the PMN surface. Normally MPO is buried behind two membranes, the cytoplasmic membrane and the granule membrane. Therefore, it cannot be stained in resting cells. Here the antigen MPO is on the outer surface of the cell. A communication has been established from granule matrix to cell surface. Antibody to lactoferrin was conjugated to rhodamine and used to stain the same cells. The results in the far right panel show that a little lactoferrin also found its way to the cell surface. The nuclear staining is nonspecific and due to methyl green which fluoresces dark red in ultraviolet light. It is used for orientation.



Figure 8. Human polymorph phagocytizing Escherichia coli 5 minutes after bacteria were added to cell monolayer. Left is a phase contrast view showing bacteria just within a space visible between nuclear lobes. Middle, the fluorescein conjugate of anti-human myeloperoxidase shows that myeloperoxidase has emerged onto the external surface of the cell membrane. Right, double staining with a rhodamine conjugate of anti-human lactoferrin shows that a small amount of lactoferrin is also on the cell surface. The same cell is seen in all three panels. It was fixed with 1% paraformaldehyde. This fixative leaves the cell membrane impermeable to the immunoglobulins of the fluorescent conjugates. Thus only antigen on the outside of the cell surface is stained.

These immunocytochemical results suggest that the mouth of the nascent phagolysosome was open, wide open, to the fluid phase and that the granules joined or fused their membranes with the phagolysosome membrane. To investigate this further we have, with Dr. Edith MacRae, used scanning electron microscopy to evaluate events on the phagocytizing cell surface. Figure 9 shows that indeed the phagolysosome where you see E. coli about to enter does gape widely, offering ample opportunity for exocytosis of the enzymes from the phagolysosome.

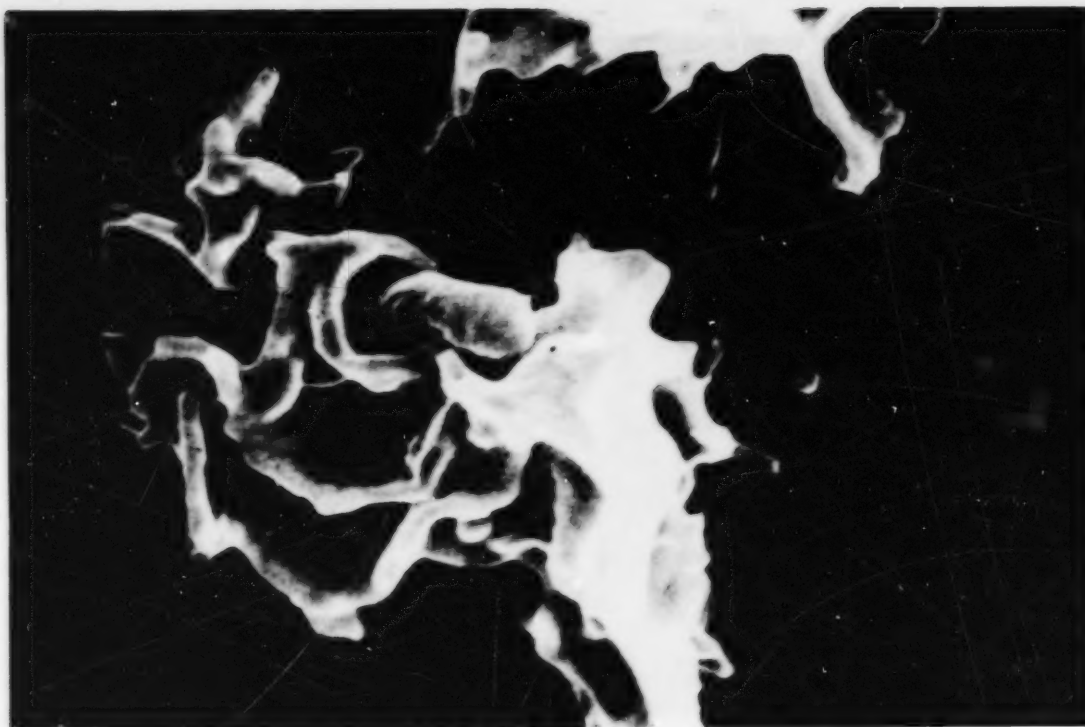


Figure 9. Human polymorph phagocytizing Escherichia coli 5 seconds after bacteria were added to the cell monolayer. Note the gap between the lips of the nascent phagolysosome and the bacterial envelope.

Findings from our laboratory and from the literature lead us to conclude that the PMN have potent enzymes capable of digesting both elastic fibers and collagen fibers. These enzymes do leave the cells, but whether they react with the connective tissues and alter them remains to be shown. It is also clear that phagocytosis and stimulation with several soluble mediators and chemicals may also induce release (2). Smoke particles with toxic chemicals absorbed to their surfaces are reported to be chemotactic and to stimulate phagocytosis (10). Even in the normal mode of antimicrobial phagocytosis granule enzymes are exocytosed onto the PMN surface and they can dissolve in the fluid phase bathing the cells. The azurophil granule contents containing elastase may conceivably within suitable localized spaces evade inhibition by α_1 -antitrypsin inhibitor and by α_2 -macroglobulin. If this happens, the enzyme could then bind to available elastic fibers within the space and damage them. Whether the collagenase or, rather, the pro-collagenase of specific granules is activated extracellularly under these circumstances is not known. Even if collagenase has no role, the elastase is

now known to depolymerize collagen (see above). This depolymerization would be expected to weaken the collagen (16).

If proteolytic enzymes generally tend to be exocytosed by phagocytizing PMN, why do they not cause damage at all times? For example, why doesn't the PMN response in pneumococcal pneumonia autolyze the lung? It is probably because biological control is available, perhaps through the antiproteases of plasma (6), perhaps through mechanisms yet to be discovered. Most important among known mechanisms with respect to PMN neutral proteases are plasma α_1 -antitrypsin and α_2 -macroglobulin. But there are, in addition, plasma α_1 -antichymotrypsin, plasma β_1 -anticollagenase, and plasma antileukoprotease. That α_1 -antitrypsin may indeed afford critical control and prevent proteolytic damage to lungs is suggested by the studies on the relationships between homozygous α_1 -antitrypsin deficiency and pulmonary emphysema. Finally, it should be noted there are antiproteases in bronchial secretions as well as in plasma.

Experimental results recently reported from Janoff's laboratory (8) suggest that cigarette smoke concentrate interferes with antiprotease activity due to pure human serum α_1 -antitrypsin, challenged with pure human PMN elastase and porcine pancreatic elastase. Moreover, Galdston *et al.* (5) have reported that pack years of smoking, elastase-like esterolytic activity of polymorphs plus trypsin inhibitory activity of plasma seem to account for 68% of variability in pulmonary function tests observed in MZ and ZZ α_1 -antitrypsin phenotypes with chronic obstructive pulmonary disease. One must conclude that the evidence that proteolytic enzymes could be factors in pulmonary damage is highly suggestive; whether these enzymes are from PMN remains to be seen.

SUMMARY

The principal need is for direct, rigorous demonstration that PMN, charged with elastase, are recruited to the strategic part of pulmonary structure in sufficient numbers, and with appropriate kinetics to account for low grade but progressive long-term damage to connective tissue, especially to elastin and possibly to collagen. Moreover, it will be necessary to show the nature and existence of conditions that lead to exocytosis of the proteases from polymorphs, that lead to the failure of biological controls due to antiproteases, and that promote degradative enzyme interactions with connective tissue fibers.

ACKNOWLEDGMENT

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DISCUSSION

DR. JAKAB: Polys are a kind of a kamikaze cycle, and their half-life is relatively short in the blood. When they get into the lung, is it necessary for them to be stimulated for the release of the enzymes or could it be that they just die and release their enzymes and result in the damage?

DR. SPITZNAGEL: It is surprising, but when you look at sections of normal lung, there are few polymorphonuclear leukocytes. This is certainly not true, for example, in the mucosa of the gut wall. That is full of polymorphonuclear leukocytes at all times, and you can even see them working out into crevices in the mucosa of the gut. There are not the large numbers of macrophages that Joe was talking about earlier with reference to lung. Polys require some specific stimulus to bring them to the lung and to keep them there. Apparently, microbial cells being in large numbers, as in pneumonia, will do this, and Kilburn has shown that instillation of cigarette smoke particles will also attract polymorphs to lung. Unless direct quantitative studies can show that polys are arriving in lung tissue and perishing very rapidly over a relatively long period of time, it is difficult to imagine polys as the major source of destructive enzyme.

Bronchus-associated Lymphoid Tissue and the Source of Immunoglobulin-containing Cells in the Mucosa

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Although the lungs are not usually regarded as primary lymphoid organs, it may be of interest to recall that in 1958 Humphrey and co-workers (1, 11) showed that following intravenous hyperimmunization with pneumococcal antigens, the lung tissue was the predominant source of specific antibodies, surpassing even the bone marrow, spleen, and lymph nodes. One explanation for these findings may have been that the lungs contained a substantial population of immunocompetent cells present by either direct immigration from the circulation or by local proliferation of a precursor population. Also, it appears from this work that the lungs can respond well to antigens introduced parenterally.

A few years ago we observed the presence of follicular lymphoid aggregates in the mucosa of the rabbit bronchial tract with a peculiar epithelial relationship (4, 5). These lymphoid follicles can, with practice, be discerned as dense white patches under the dissecting microscope or upon treatment of the respiratory tract with 2% acetic acid. The distribution of this lymphoid tissue is random along the length of the bronchial tract as far as the small bronchioles and seems to be concentrated around bifurcations. The tissue is present in all mammals examined so far with the possible exception of the Golden hamster. Chickens also possess considerable amounts of this tissue which projects into the lumens of the large bronchi. Morphologically, this bronchus-associated lymphoid tissue (BALT) was remarkably similar to the Peyer's patches of the gut. Interestingly, Klein in 1875 (13) wrote, "...these lymphoid follicles of the bronchial walls are, therefore, in every respect analogous to the lymph follicles found in other mucous membranes, e.g. tonsils and in the intestine."

The epithelium which overlies the BALT follicles is unlike that found elsewhere in the bronchus and is constructed of flattened, irregular shaped cells. Often the BALT epithelium is heavily infiltrated with lymphocytes from the underlying follicles (3). This lymphoepithelium is reminiscent of the follicle-associated epithelium overlying Peyer's patches in the gut (6) and is deficient in glandular tissue and goblet cells as well. In addition, cilia are irregularly distributed and ultrastructural examination reveals the presence of microvilli. Rare plasma cells are seen scattered throughout the lymphoid follicles and occasionally plasma cells are found in the overlying lymphoepithelium (20). The follicles contain the high-walled endothelium associated with postcapillary venules, and this type of endothelium appears to be more prominent following antigen challenge (20).

The lymphoepithelium seems to act as a sampler of the luminal antigen much as does the follicle-associated epithelium of the Peyer's patches and also the bursa of Fabricius (6). Horseradish peroxidase introduced into the lumen of the rabbit tracheobronchial tree is selectively taken up by the lymphoepithelium overlying the BALT follicles (26). A fairly extensive proliferation of cells in the follicles occurs after antigen challenge, particularly with *Bacillus Calmette-Guerin* (20), and following in vivo injections of tritiated thymidine, heavily labeled cells are seen 24 hours later in a crescentic manner capping the luminal aspects of the follicles (5). However, the BALT seems to develop in the virtual absence of antigen. When fetal pulmonary tissue was transplanted to ectopic subcutaneous sites in syngeneic adult mice, BALT follicles still developed but appeared immature (5, 15). The fact that germ-free animals possess a BALT mass considerably less than that seen in conventional animals strongly suggests that antigen promoted complete BALT development (4).

The predominant types of cells present in BALT, as judged by surface membrane antigens, are neither T nor B cells (24). In young adult rabbits, 18.4% of BALT cells bore a thymic antigen whereas in Peyer's patches and intestinal lamina propria, this figure was 16.6% and 11.1%, respectively. Approximately 40% of BALT cells bore surface immunoglobulin and 17.2% of these had surface IgA, a value intermediate between that of Peyer's patches (29.9%) and gut lamina propria (7.3%). The finding of BALT intermediacy between the Peyer's patches and the intestinal lamina propria provides some clues as to the functions of the BALT.

Some evidence indicates that the BALT is functionally similar to the Peyer's patches. It has been demonstrated (25) that cells derived from the BALT had virtually the same capacity as Peyer's patch cells to repopulate the spleen of irradiated allogeneic recipients with IgA-containing cells. Of greater significance, cells derived from the BALT were nearly as effective as Peyer's patch cells in repopulating the lamina propria of the bronchus and gut with IgA plasma cells.

These findings demonstrated that cells from one mucosal site could move and perhaps differentiate at another mucosal site, and supported our original hypothesis, in 1973, that the BALT might be part of a more universal mucosal lymphoid system (4).

In 1974, we proposed that there may be a common mucosal immunologic system (2). Montgomery (16) has shown that rabbits orally immunized with DNP-pneumococci have a selective appearance of specific IgA antibody in the milk in the apparent absence of such antibody in the circulation. Similar feeding experiments have been done by Hanson and co-workers in humans using a non-pathogenic E. coli (9). The subjects which had received E. coli subsequently developed a rise of specific IgA antibody against E. coli in the milk and antigen-specific IgA antibody-containing cells appeared in their colostrum. Lamm and co-workers (22) have shown that dividing cells from the mesenteric lymph node have a tendency to selectively localize in the mammary glands when adoptively transferred into syngeneic mice in late gestation. This selective migration was enhanced greatly by lactation and could be induced in nonpregnant animals by the suitable injection of sex hormones. Thus, the antibody and cells in milk specific for enteric antigens probably result from the migration of cells from gut to breast tissue. We have done similar experiments with dividing cells from the bronchial lymph nodes and mesenteric lymph nodes and have shown that these cells have a tendency to home to mucosal tissues including the breast, bronchus, bowel, and cervix where they make predominantly IgA (14). Thus, it seems likely that a common mucosal system may well exist, at least for those mucosal sites where cells are potentially primed or sensitized and have a tendency to traffic to another mucosal site.

One question which has not been completely answered is what the role of antigen might be in lymphocyte traffic between mucosal surfaces. This traffic appears to be independent of antigen although the presence of antigen, to which the cells have been primed, clearly causes a greater number of IgA cells to appear in the lamina propria of the bowel mucosa (18). It may be postulated that antigen might cause cells to divide locally or even retain cells once they have immigrated into the mucosal lamina propria. In this event, if cells were not exposed to the sensitizing antigen they might leave (presumably by lymphatics), die, or, alternatively, find their way via the mucosal epithelium into the luminal spaces. The cytokinetic experiments referred to earlier found heavily labeled cells within the lymphoepithelium of the BALT 24 hours after the in vivo injection of tritiated thymidine (5). We have chosen to interpret this finding as partial support for the suggestion that these cells may be on their way out into the lumen although no direct evidence for this exists.

Careful examination of preparations of BALT led to the appreciation that there were a number of cells present which had metachromatic granules in the cytoplasm giving the appearance of primitive mast cells (3). Since some of these cells may well have emanated from the bronchial lamina propria, a systematic attempt was initiated to look for them in BALT tissue. We did indeed find, with special stains and ultrastructural techniques, the presence of basophil-like cells, particularly in the peripheral zones of the BALT follicles. These cells were also seen above the basement membrane in the epithelium adjacent to these follicles and resembled the basophiloid cells which have been described by Patterson in several animal species in the bronchial washings (17). Collan (7) has described granular lymphocytes in the rat intestinal epithelium. The relationship of these cells to mast cells is unclear, but we have also observed such granular lymphocyte-like cells in preparations from the gut lamina propria and have identified these primarily in the epithelium in rabbits (23). Recently, Guy-Grand *et al.* (10) have made a number of observations about these cells in the small intestine and have concluded that the cells in the gut epithelium appear to arise from a special population of T cells present in the Peyer's patches. It is tempting to speculate that a similar Peyer's patch (or BALT?) derivation applies to the basophiloid cells in bronchial epithelium. Metachromatic, granule-containing cells are also found in Peyer's patches. These cells, which lie in the spaces formed between epithelial cells, are potentially in direct contact with antigen which traverses the mucosal epithelium and is then exocytosed into the lateral interepithelial spaces. Much more work needs to be done in this area to establish the relationship of epithelial basophiloid cells to T cells or mast cells and to explore the functional activity of this cell type.

There is some evidence for T blast traffic between mucosal surfaces of a similar nature to that described above for B blasts, particularly precursors of IgA (21). Relatively little is known about T blast traffic to the respiratory tract and it is an open question whether such a common mucosal system exists for T cells. Waldman and Henney (27) first showed that following intratracheal immunization, cells washed out from the lungs could proliferate and release migration inhibition factor (MIF) on exposure to specific antigen. It is unclear whether the cells from which this lymphokine was derived were T cells or B cells, especially since both types of cells will produce MIF. In any event, local immunization has been shown to produce a local cell-mediated specific immune reaction. Immunization protocols have been devised mostly in experimental animal models to prove that local immunization results in local antibody and local cell-mediated immune reactions; such reactions are more consistent and of a higher intensity than when antigen is introduced parenterally. It must be remembered when looking at these results that the lung, from the standpoint of mucosal immune responses, is divided into at least two compart-

ments, that which is mucosal and that which is peripheral and very similar to the blood in terms of the antibodies contained therein (12). If one draws an analogy with the bowel, it appears, especially from the work of Pierce and colleagues (18, 19), that parenteral immunization followed by local challenge leads to a higher accumulation of IgA antibody-producing cells in the intestine than with any other immunization protocol. It follows, therefore, that antibody and concomitant protection against a particular organism might best be achieved locally in the respiratory tract if parenteral immunization occurred first and was then followed by local presentation of antigen. The best way to prime for complete protection of the lungs against subsequent infection is not well understood.

Local immunization of the lungs may lead to local IgE antibody formation which could, of course, be deleterious. Gerbrandy and Bienenstock (8) have shown that following intratracheal immunization potential IgE-forming cells could be demonstrated in the draining mediastinal lymph nodes in greater numbers than following peripheral immunization. Intraperitoneal immunization also produced the same results, but this observation is confusing since it has been known for many years that intraperitoneal immunization is a good method of priming for the IgE response and antigens introduced into the peritoneal cavity will drain through the diaphragm into the mediastinal nodes. Obviously until the potential hazards of producing a local IgE type of immune response versus a protective immune response at a local mucosal surface are better understood, immunization protocols will perforce not be able to maximize and mobilize the appropriate immune response at the appropriate mucosal site.

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The Role of Polyclonal Cell Activation in the Initiation of Immune Complex-mediated Pulmonary Injury Following Antigen Inhalation

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Certain forms of environmental lung disease provoked by inhaled substances are thought to result from immunologically mediated inflammatory injury to the pulmonary parenchyma; such diseases have been termed "allergic alveolitis" or, alternatively, "hypersensitivity pneumonitis." To better understand the pathogenesis of such injury, animal models have been studied in which protein antigens, polyclonal cell activators derived from plants, or other organic materials have been administered in aerosol form to normal or immune animals.

Inhalation of bovine serum albumin (BSA), a protein antigen, causes little, if any, injury in BSA-immunized rabbits, presumably because it does not have access to humoral antibody (18). However, we have observed that inhalation challenge with mixtures of antigen plus concanavalin A (Con A), a polyclonal cell activator, produced a severe necrotizing pneumonitis in immune recipients, in association with localized deposits of immune complexes of inhaled antigen, host antibody, and complement (19, 20). We now present evidence that this enhancement of immune complex formation in the lung by inhaled Con A occurs as a direct consequence of Con A's ability to stimulate lymphocytes in vivo. To do this, we have employed purified cholera toxin (CT), a cAMP agonist which inhibits leukocyte stimulation by Con A in vitro.

First, we have compared three different regimens of in vivo CT administration for inhibition of Con A-induced stimulation of peripheral lymphocytes in vitro, and also for inhibition of Con A-induced inflammation in the

skin. We then demonstrate that CT administration selectively inhibits cell-mediated, delayed hypersensitivity skin reactions but not the antibody-mediated vasculitis associated with the Arthus reaction. Finally, we show that when we block cell-mediated reactivity to Con A in vivo, we also block the ability of Con A to trigger immune complex reactions in the lung between inhaled antigen and humoral antibody. Lastly, these inhibitory effects of CT can be correlated with elevations in lymphocyte cAMP concentrations associated with CT administration.

MATERIALS AND METHODS

New Zealand White male rabbits used in this study weighed 2-3 kg and were purchased from Hare (Hare, N.Y.). The rabbits were housed in laminar flow rooms supplied with filtered air and given both rabbit chow and water ad lib. All animals were immunized with BSA (Sigma, St. Louis, Mo.) incorporated into an alum precipitate as previously described (18). Each rabbit received a total of 50 mg BSA, 10 mg of protein initially, followed by 20 mg each on days 7 and 21, injected bilaterally in four equal parts at dorsal and gluteal sites.

Aerosols containing BSA and Con A were administered as previously described (18). Briefly, aerosols were generated in a DeVilbiss model 35A ultrasonic nebulizer containing a solution of 100 mg BSA and 100 mg Con A in 20 ml of PBS. Each aerosol was vented into a Plexiglas chamber into which two rabbits' heads could be inserted simultaneously. Each aerosol administration required approximately 60-90 minutes to complete, and was administered to each animal on 3 consecutive days. All animals were then sacrificed 24 hours after the last aerosol by an intravenous overdose of nembutal.

Concanavalin A

Con A was prepared from Jack bean meal (Difco, Detroit, Mich.) according to the method of Agrawal and Goldstein (1), modified as previously described (20). The purified Con A gave a single precipitin band by immunodiffusion in gel against rabbit anti-Jack bean meal antibody, and a single symmetrical peak by velocity sedimentation in a 10-40% sucrose density gradient.

Cholera Toxin

CT was purchased from Schwarz/Mann (Orangeburg, N.Y.). It was characterized by the supplier as giving a single band in disc electrophoresis. One milligram of lyophilized CT was initially resuspended in 1 ml H₂O, then diluted in saline to a final concentration of 100 µg/ml or 50 µg/ml. Prior to injection, the diluted CT was filtered through a 0.22 Millipore filter

(Bedford, Mass.). In search for optimal conditions of inhibition, several different regimens of CT administration were employed in initial studies. In some experiments (protocol A), rabbits were injected via the marginal ear vein with 40 μ g of CT/kg, 24 hours prior to the first aerosol, followed 48 hours later with a second intravenous injection of 20 μ g/kg. In others (protocol B), daily injections of equal amounts were given on four consecutive days, beginning 24 hours prior to the first aerosol; the total daily dosage in the latter protocol was 20 μ g/kg body weight or approximately 160 μ g/animal. Finally, a third group of rabbits (protocol C) was given 40 μ g of CT/kg intraperitoneally, followed 48 hours later with a second injection of 20 μ g/kg.

The amounts of CT employed in these studies were derived empirically in preliminary experiments. It is apparent from such studies that the amounts of CT required for suppression of immune effector reactions in vivo are near lethal values. Although no rabbit died in the experiments presented here (nor in most subsequent experiments carried out to date), we have on occasion seen animals receiving similar amounts of CT die within 24-48 hours of the first injection. At autopsy, the only obvious pathological finding in these animals was the occasional presence of fluid in the pleural cavities.

Lymphocyte Cultures

Rabbits were bled from the central artery of the ear and the peripheral lymphocytes isolated by the method of Boyum (2). The cells were washed twice with Hanks' balanced salt solution (HBSS) (Microbiological Associates, Rockville, Md.) and, if necessary, any remaining red blood cells lysed with 0.84% ammonium chloride. The lymphocytes were diluted in culture medium to give a suspension of 2×10^6 cells/ml. The culture medium employed consisted of RPMI 1640 (Microbiological Associates) containing 10 mM HEPES buffer (Sigma, St. Louis, Mo.), 10% heat inactivated pooled normal rabbit serum, 2 mM glutamine, 100 units/ml penicillin, and 100 μ g/ml streptomycin (Microbiological Associates).

Equal volumes (0.1 ml each) of diluted cells (2×10^5 cells) and of medium containing Con A were added to each well of microculture plates (Costar Plastics, Cambridge, Mass.). After incubation for 52-54 hours at 37°C in humidified air containing 5% CO₂, 0.05 ml of media containing 0.5 μ Ci of ³H-thymidine (specific activity = 5.0 Ci/mM, Amersham/Searle, Arlington Heights, Ill.) was added to each well. After 72 hours of total incubation time, the cells were harvested on Reeve Angel glass fiber filters (Whatman Inc., Clifton, N.J.) with an automatic cell harvester (Model 24V, Brandel, Rockville, Md.). The filters were air dried and then placed in plastic scintillation vials (MiniVials, RPI, Rochester, N.Y.). Five milli-

liters of scintillation fluid (4 g Omniscint to 1 L toluene, RPI) were added to each vial and the samples counted in a Beckman LS 250 or LS 7000 liquid scintillation spectrophotometer. The degree of lymphocyte stimulation is expressed as the net cpm (total cpm minus cpm of unstimulated control cultures). Data are reported as the mean of triplicate cultures $\pm$ standard error of the mean.

Cyclic Nucleotide Determinations

Lymphocytes were isolated from arterial blood as described above and 0.5 ml aliquots containing 2×10^6 cells in HBSS were placed in 16 x 125 mm Pyrex test tubes, frozen rapidly in alcohol and dry ice, and then stored at -60°C . For assay, the cells were thawed slowly, 3 ml of ice-cold acetone added, and the cell suspension then allowed to stand for 15 minutes in an ice bath. The cell suspensions were then heated in a $60-70^\circ\text{C}$ water bath for 3 minutes followed by centrifugation at $900 \times g$ for 15 minutes at $2-5^\circ\text{C}$. The supernatants were transferred to Pyrex culture tubes and lyophilized.

Radioimmunoassays (RIA) for cAMP and cGMP were carried out under identical conditions, as previously described (10), using commercial reagents (Schwarz/Mann, Orangeburg, N.Y.). Briefly, lyophilized samples were resuspended in 1.0 ml of cold ($2-5^\circ\text{C}$) 0.05 M sodium acetate buffer (pH 6.2) and 0.3 ml transferred to each of two 12 x 75 mm polypropylene culture tubes (one for cAMP and one for cGMP), followed by the addition of 0.1 ml of radioactive cyclic nucleotide [2-O-succinyl cyclic AMP (or cGMP) tyrosine methyl ester (^{125}I)] and 0.1 ml of rabbit IgG anti-cAMP or anti-cGMP to each tube. After mixing, all RIA tubes were held at 4°C for 16-20 hours, followed by addition of 2.5 ml of 60% saturated ammonium sulfate to each tube. Thirty minutes later, the tubes were centrifuged at $900 \times g$ for 30 minutes at $2-5^\circ\text{C}$. The supernatants were decanted and any excess fluid removed with a cotton-tipped applicator. The precipitate radioactivity was measured in a Beckman 310 gamma spectrophotometer; the efficiency of ^{125}I activity measurements was determined to be 75%. For each assay, standard curves were constructed using known amounts of cyclic nucleotides (ranging from 0.05 to 10.0 pmoles/tube). These curves were then used to relate the amount of cyclic nucleotide in unknown samples as a function of precipitated radioactivity.

Skin Tests

Rabbits were skin tested 20-24 hours prior to sacrifice. All skin tests were done on the animal's left side, which had been previously shaved. Arthus skin tests were induced by intradermal injection of 0.05 ml of BSA dissolved in PBS at a final concentration of 1 mg protein/ml. Cell-mediated inflammatory skin reactions were induced by intradermal injection of 0.05 ml

of Con A (200 µg Con A/ml PBS). In some instances, where negative reactions were anticipated, sterile india ink (1%) was added to the Con A and BSA skin test solutions. The india ink provided a marker for the positive identification of non-inflammatory skin test sites; by itself, the ink caused no inflammatory response. After sacrifice, each test site was excised and fixed in formalin for subsequent processing for histologic examinations.

Histology

All tissue was fixed in formalin and paraffin-embedded tissue sections stained with hematoxylin and eosin. Lungs were inflated with isotonic buffered dormalin at a hydrostatic pressure of 10 cm H₂O. Sections were taken from all lobes; each section extended from the hilar region out to the periphery. Injury was judged and graded on the extent or degree of inflammatory change, and also in terms of the presence or absence of parenchymal necrosis.

RESULTS

Effects of CT on Peripheral Blood Lymphocytes (PBL)

The effects of CT in vivo were examined using three alternative administrative protocols. In protocol A, a total of 60 µg CT/kg of body weight was injected intravenously, two-thirds being given at time 0 and the remaining one-third at 48 hours; in protocol B, a total of 80 µg CT/kg of body weight was injected intravenously, given daily, starting at time 0, as four equally divided doses; in protocol C, a total of 60 µg CT/kg body weight was injected intraperitoneally, two-thirds at time 0 and the remaining one-third at 48 hours. Peripheral blood lymphocytes were isolated from bleedings obtained at daily intervals, and tested for their ability to incorporate ³H-TdR when stimulated with Con A in vitro.

All animals given CT intravenously showed a drop in PBL responsiveness within 24 hours following the initial injection (Fig. 1). Animals treated with CT via protocols A or B (intravenously) showed over a 90% inhibition in proliferative responses to Con A, and remained unresponsive throughout the remainder of the experiment. On the other hand, administration of CT via the intraperitoneal route was notably less effective, in terms of both the rapidity of onset as well as the extent of inhibition observed.

Effects of CT Administration on Con A-induced Cutaneous Inflammation

The ability of Con A to activate T-lymphocytes (and basophils) has been demonstrated in vivo as well as in vitro; intradermal injections of Con A induce a diffuse, local inflammatory reaction, predominantly mononuclear in

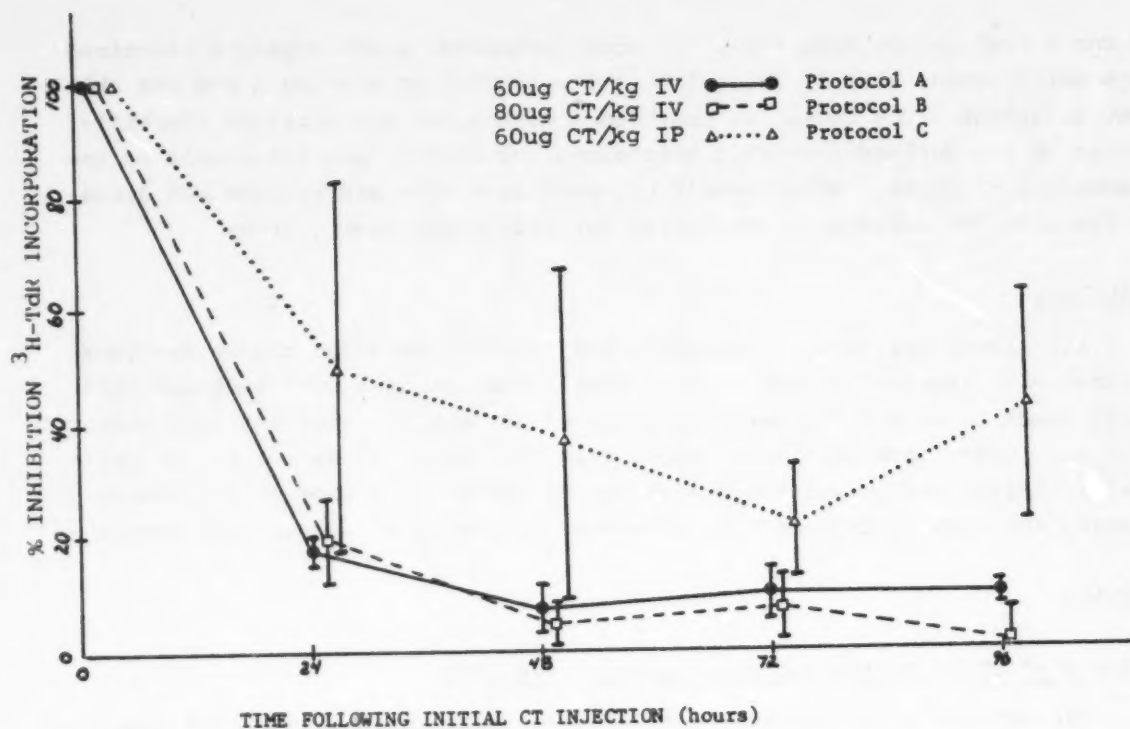


Figure 1. Effects of CT administration in vivo on responsiveness of peripheral blood lymphocytes (PBL) to stimulation in vitro by optimal concentrations of Con A. Three different protocols (A, B, and C) for CT administration have been employed and their effects compared. Results are plotted as percent of ^3H -TdR incorporation observed prior to CT administrations (time = 0), as observed in PBL obtained 24, 48, 72, and 96 hours following the initial CT injection. Each point represents the mean value observed, using triplicate assays, in each of three animals; vertical bars express the standard error.

character, and resembling the classic tuberculin-type, delayed hypersensitivity reaction (8, 9, 13). Therefore, we examined the ability of CT to inhibit Con A-induced cutaneous inflammation, as might be expected if CT inhibited leukocyte activation in vivo as well as in vitro.

Ten micrograms of Con A were injected at duplicate sites in rabbits receiving CT according to protocols A (3 animals), B (3 animals), or C (3 animals), and also into non-CT treated controls (3 animals). Both schedules of intravenous CT administration (A and B) effectively inhibited the Con A-

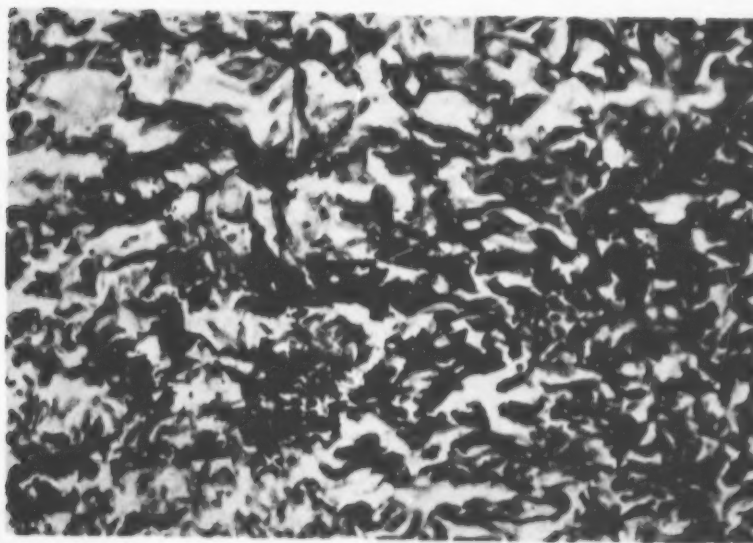
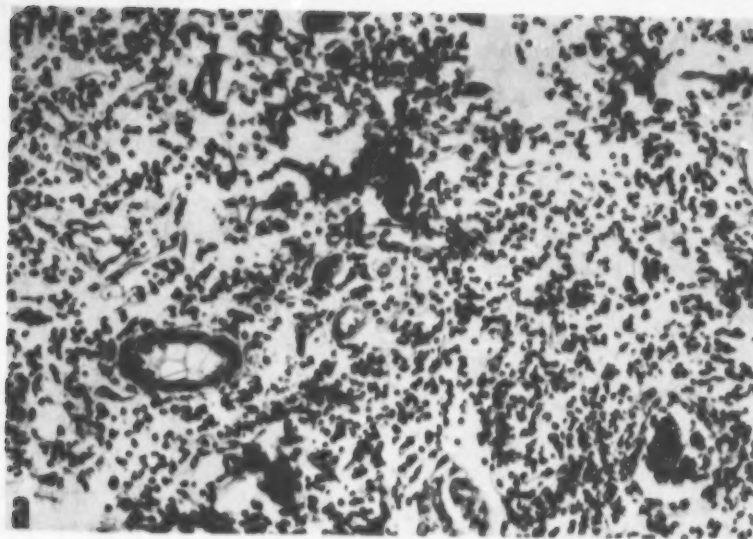


Figure 2. Local inflammatory response to intradermal injections of Con A in the rabbit: a) Diffuse inflammatory infiltrate observed in the collagenous dermis of a normal rabbit 24 hours following injection of 10 μ g Con A (X155); b) Absence of any inflammatory reaction 24 hours following injection of 10 μ g Con A in a CT-treated rabbit (protocol A). The black particulate material present in the center of the field is sterile india ink which had been admixed with the Con A to positively identify the injection site (X155).

Table 1. Effects of CT Administration on Con A-induced Cutaneous Inflammation

Rabbit No.	CT administration protocols	Skin test grade ^a (duplicate sites)	
293	(A) 40 µg/kg IV, day 0,	+	++
294	20 µg/kg, 48 hr later	+	0
295		+	0
296	(B) 20 µg/kg IV, daily	0	0
297	for 4 days	+	0
298		0	0
299	(C) 40 µg/kg IP, day 0,	+++	++
300	20 µg/kg, 48 hr later	+++	++
288		+++	+++
249		++++	++
251	None	+++	++++
252		++++	++++

^aIntensity of inflammation graded from 0 (no inflammation) to ++++ (extensive inflammation).

induced inflammatory response (Fig. 2), whereas intraperitoneal administration gave little, if any, inhibition (Table 1). These results paralleled those obtained in the preceding experiment, again indicating that, whereas intravenous administration of CT effectively blocked *in vivo* the lymphocyte's responsiveness to Con A, intraperitoneal administration was decidedly less effective. Based on these observations, protocol A was employed in subsequent experiments.

Differential Effects of CT Administration on Delayed Hypersensitivity and Immune Complex Reactions in the Skin

The direct active Arthus reaction, induced by injecting antigen intradermally into an actively immunized animal, consists in part of a necrotizing vasculitis mediated by immune complexes of antigen, antibody and complement, localized within the vessel wall, and, in part, of a diffuse cutaneous leukocytic infiltrate (similar to that produced by Con A) considered to represent a delayed-type hypersensitivity response (3). Importantly, the vasculitis does not require participation by sensitized T-lymphocytes, but instead is mediated by immune complexes, together with homocytotropic antibodies which

Table 2. Differential Effects of CT Administration on Delayed Hypersensitivity and Immune Complex Reactions in the Skin^a

Rabbit No.	Total μg CT/kg body weight	Arthus reaction (50 μg BSA)		
		Vasculitis	Diffuse inflammation	Delayed hypersensitivity (10 μg Con A)
133	0	++++	++++	++++
134	0	++++	++	++++
138	0	+++++	+++	++++
140	0	+++	++	+++
135	60	++++	0	++
136	60	++++	0	++
137	60	++++	0	++
139	60	+++++	0	0
141	60	++++	0	+

^aIntensity of inflammation graded from 0 (no inflammation) to +++++ (extensive inflammation).

participate in initiation of complex formation, and neutrophils which respond to the complement-mediated chemotactic stimulus (3). Thus, it can be readily produced in animals immunized passively with antibody alone.

Using both the BSA-induced Arthus reaction (in BSA-immunized rabbits) as well as the Con A skin test, we were thus able to compare the effects of CT administration upon an immune complex-mediated vasculitis with its effects upon delayed-type hypersensitivity reactions.

As in the previous experiment, intravenous administration of CT inhibited the inflammatory response to Con A (Table 2). Of particular interest, however, was its effect upon the active Arthus reaction, where it inhibited the diffuse inflammatory or delayed hypersensitivity component of the Arthus, but not the immune complex-mediated vasculitis (Fig. 3). Thus, these findings argue eloquently for a selective inhibitory effect by CT upon effector lymphocytes of the delayed-type hypersensitivity reaction, while leaving simple immune complex-mediated reactions intact.

Effects of CT upon the Immunologic Pulmonary Injury Associated with Inhalation of Antigen Plus a Polyclonal Cell Activator

We have recently reported that polyclonal cell activators such as Con A

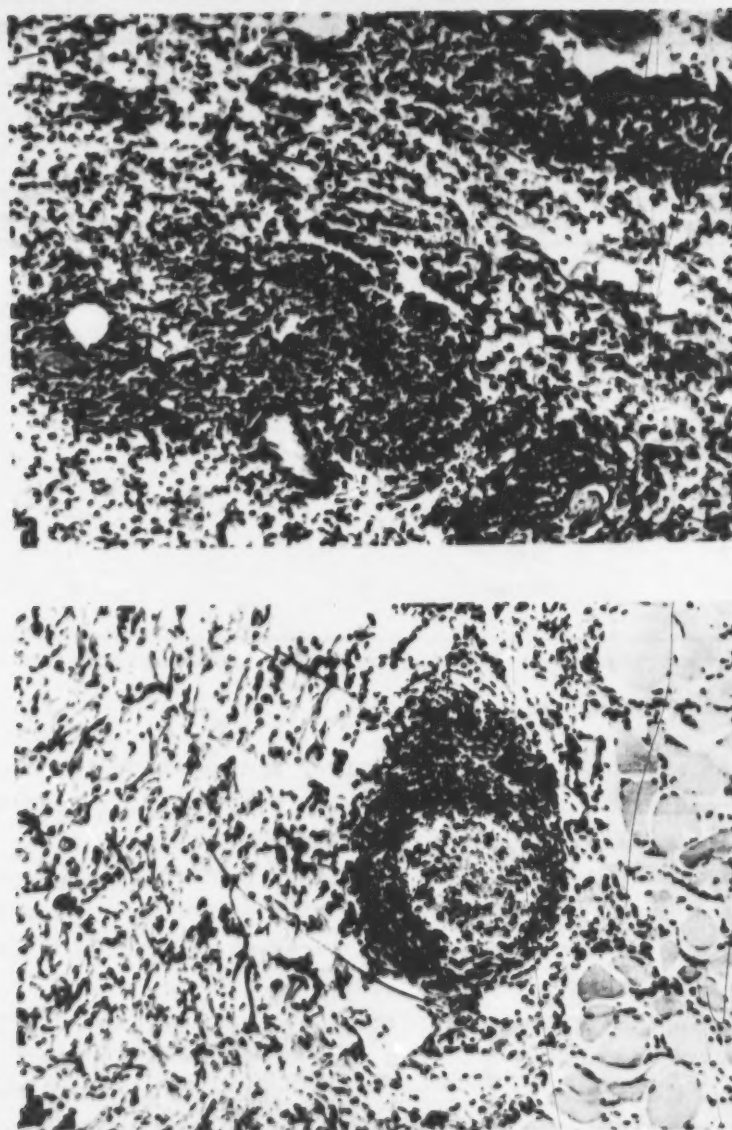


Figure 3. Direct active Arthus reaction to BSA injected intradermally into a BSA-immunized rabbit. a) Arthus reaction in a control animal. Notice both the necrotizing vasculitis (mediated by immune complexes containing BSA, anti-BSA antibody, and complement) and the diffuse inflammatory infiltrate (representing a delayed hypersensitivity reaction to BSA) (X155). b) Direct active Arthus reaction in a rabbit treated with CT. An intense necrotizing vasculitis is still seen, but the diffuse inflammatory component is notably reduced (X155).

and phytohemagglutinin induce a diffuse interstitial pneumonitis when inhaled in aerosol form (19). More importantly, they also exert a pronounced enhancing effect upon immune complex injury involving inhaled antigen, humoral antibody, and complement; such injury is severe and is characterized by foci of frank parenchymal necrosis (20). We have proposed that this enhancement may be mediated, at least in part, through polyclonal activation of leukocytes within the lung by Con A, leading in turn to the interstitial inflammation typically observed following inhalation of Con A (20). In particular, Con A is known to cause T-lymphocytes to release vascular permeability factors (12) in vitro, and to cause mast cells and basophils to release vasoactive amines in vitro (11); inhaled Con A could presumably be having the same effect on these cells in vivo. In any event, inhaled Con A might alter the cellular barriers which normally isolate intra-alveolar antigen from humoral antibody and complement. If this hypothesis is true, then administration of CT should block both Con A-induced interstitial pulmonary inflammation and immune complex-mediated parenchymal necrosis, even though, as seen in the preceding experiment, CT has no apparent effect on simple immune complex-mediated reactions such as the Arthus vasculitis.

The BSA-immunized rabbits were divided into two groups of five each; one group was given CT intravenously, according to protocol A; the other group did not receive CT but served as a control. All animals were challenged with Con A/BSA aerosols at 24, 48, and 72 hours following the initial CT injection, and were sacrificed at 96 hours. Histologic examinations of lung tissues revealed that both the extent and severity of inflammation were greatly reduced in the CT-treated animals as compared to the controls (Fig. 4). Of particular importance was the striking reduction in the incidence of immune complex-type necrosis, with such lesions being observed in only two lobes from a single animal receiving CT, whereas such lesions were present in a total of 14 lobes of the control group, with all animals being affected (Table 3).

In vitro studies have shown CT to act through adenylate cyclase to increase cellular cAMP levels. In order to look for a similar effect in vivo, we obtained daily bleedings from the five rabbits receiving CT via schedule A, as well as from the five controls, harvested the lymphocytes and measured their cAMP levels by radioimmunoassay (Table 3). The average cAMP concentration in cells obtained following the initial CT injection was 18.6 ± 2.4 pmoles/ 10^6 cells, significantly greater than that in cells from control animals (7.0 ± 1.2 pmoles/ 10^6 cells, $P = 0.002$).

In some cases, different experimental observations were carried out simultaneously in the same animals, thereby providing a form of internal control and underscoring the mutual relevance of the different phenomena. For

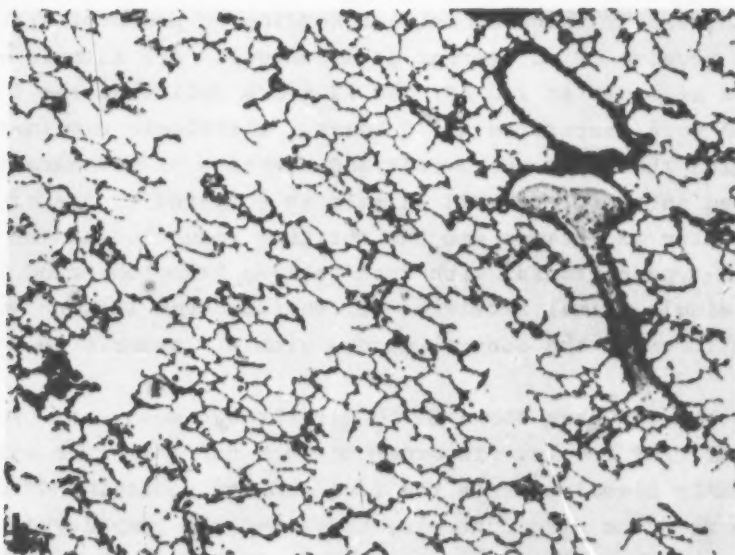
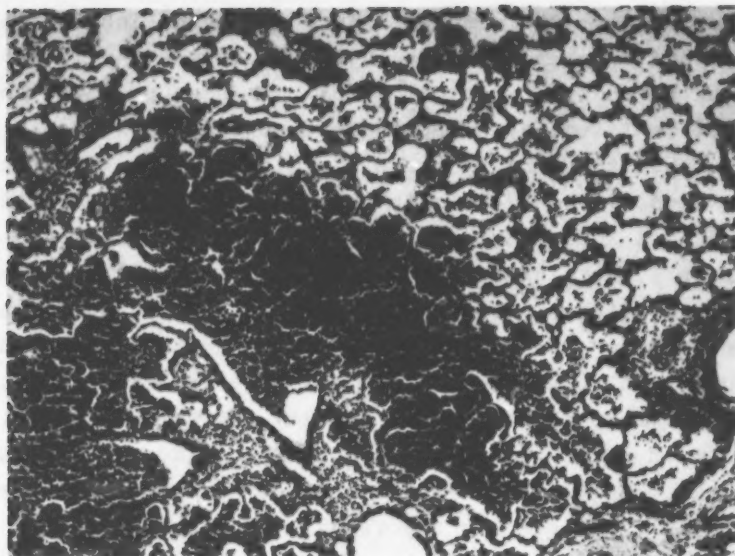


Figure 4. Pulmonary injury following administration of BSA/Con A aerosols to BSA-immunized rabbits. A) Severe interstitial pneumonitis with areas of frank parenchymal necrosis in a control animal not given CT (X60). b) Inhibition of pulmonary injury in a CT-treated animal. A small focus of interstitial inflammation is seen in the right upper corner (X60).

Table 3. CT-induced Suppression of Immunologic Pulmonary Injury^a in BSA-immunized Rabbits Challenged with BSA/Con A Aerosol

Rabbit No.	μ g CT/kg body wt.	MeL ^b	Lung injury						Lymphocyte cAMP (pmoles/ 10^6 cells)
			RUL	RML	RL _L _h	RL _L _p	LUL	LL _L _h	LL _L _p
131	0	0	++++	++++	+	0	+++	++	0
133	0	0	0	++	++	+	+	0	0
134	0	+++	+	++++	+	0	++++	0	0
138	0	++++	++++	++++	++	++	++++	+	0
140	0	0	+	+++	0	0	++++	0	+
135	60	+	0	++	0	0	+	0	0
136	60	0	0	+	++	0	0	ND	0
137	60	++++	++	++++	++	0	++++	+	0
139	60	+	0	+	0	+	0	+	0
141	60	0	0	++	0	0	+++	0	0

^aLesions graded from 0 (no injury) to ++++ (severe injury). Lobes containing one or more foci of parenchymal necrosis are enclosed in boxes. ND = not examined.

^bMeL = mediastinal lobe; RUL = right upper lobe; RML = right middle lobe; RL_L_h = hilar portion of right lower lobe; RL_L_p = peripheral portion of right lower lobe; LUL = left upper lobe; LL_L_h = hilar portion of left lower lobe; LL_L_p = peripheral portion of left lower lobe.

example, the selective action of CT upon cell-mediated versus antibody-mediated reactions in the skin, its effects on Con A/BSA aerosol challenge, and on cAMP levels in peripheral blood lymphocytes, were examined simultaneously in animals 131-141 (see Tables 2 and 3). Thus, observations that CT treatment was able to block the immune complex-mediated parenchymal necrosis, but not the Arthus vasculitis, were made simultaneously in individual CT-treated animals.

DISCUSSION

We have previously proposed that inhaled polyclonal cell activators enhance local immune complex injury, in an indirect fashion, through their ability to stimulate pulmonary lymphocytes. These activated lymphocytes could then "trigger" immune complex formation between intra-alveolar antigen and humoral antibody by releasing lymphokine mediators or, possibly, through direct cell-cell cytotoxic reactions. This hypothesis has been critically tested in this study by examining whether selective inhibition of lymphocyte responses to Con A in vivo in fact blocked not only cell-mediated interstitial lung injury, but immune complex-mediated parenchymal necrosis as well.

To achieve such a selective inhibition in vivo, we elected to use CT for several reasons. First, the ability of CT to inhibit lymphocyte effector functions in vitro through its actions as a cAMP agonist is now well documented (15, 16). Moreover, we favored CT over other known cAMP agonists since it binds irreversibly to cell membranes, and its cAMP stimulating effects are notably prolonged relative to those of the beta adrenergic stimulators (14). In addition, Warren and colleagues (17) had previously reported that CT, when administered intravenously to mice, inhibited cell-mediated inflammatory responses to schistosome egg antigen, in association with a doubling of spleen cell cAMP content.

As a first step, we demonstrated that CT administration could inhibit peripheral blood lymphocytes. Lymphocytes harvested from CT-treated animals were shown to have impaired proliferative responses when treated with optimal concentrations of Con A in vitro, and the local in vivo inflammatory response to intradermal injections of Con A was also suppressed. Second, we showed that the effect of CT on immune effector responses was selective, by demonstrating that it inhibited both the cell-mediated response to Con A and the classic delayed-type hypersensitivity response to the protein antigen, BSA, but that it failed to inhibit the cutaneous Arthus vasculitis mediated by antigen, antibody, and complement. The latter is particularly noteworthy, since there is good evidence that this vasculitis is triggered by an anaphylactic reaction involving the release of vasoactive amines (and possibly platelet activating factor) from mast cells and basophils sensitized with

homocytotropic antibody (3). Thus, the inability of CT to block the Arthus vasculitis also argues against any significant inhibition of basophil or mast cells by CT in these studies.

The present demonstration that CT administration blocks both the interstitial pneumonitis [associated with injury by Con A alone (20)] and the immune complex-associated necrotizing injury supports the hypothesis that cell-mediated lung injury acts to enhance or facilitate immune complex formation in the lung. Stimulation of T-lymphocytes appears to be the critical initiating event for the following reasons: 1) Con A is known to directly stimulate T cells (but not B cells) (6); 2) T-lymphocytes are normally present in the lung (4); 3) Con A has little effect upon macrophages (5); and 4) we have now demonstrated that inhibition of immune complex lung disease by CT is associated with inhibition of T-lymphocyte functions, in the absence of demonstrable effects on the immune complex-mediated, basophil/mast cell-initiated Arthus vasculitis.

It should be noted that in some instances the cutaneous response to Con A and also the production of interstitial pneumonitis were only partially blocked by CT, yet inhibition of immune complex injury was striking. Thus, initiation of immune complex injury seems dependent upon the extent of cell-mediated injury produced. Similarly, this finding may also explain the relative inability of BSA alone to initiate immune complex injury (although cell-mediated responses to BSA were observed by the direct active Arthus skin test) since only a small percentage of bronchoalveolar T-lymphocytes would theoretically specifically recognize BSA antigen. In contrast, all or most T-lymphocytes should respond to polyclonal activation by Con A except, of course, in animals treated with CT.

Demonstrations of significant increases in cAMP in peripheral lymphocytes from CT-treated animals indicate that CT is exerting its inhibitory effects in vivo through stimulation of intracellular "second messengers." This finding parallels those changes observed when such cells are treated with CT in vitro (15, 16), and the 2.7-fold increase observed here agrees well with the value of 2.1 that Warren and colleagues observed in mouse spleen cells following a single injection of CT (17).

In conclusion, these studies call attention to the possible importance of polyclonal cell activators which, when present in the environment, may play a critical role in initiating environmental lung disease. Such polyclonal activators include not only a vast array of plant proteins, including lectins such as that employed in this study, but also endotoxins, enzymes, and even simple inorganic chemicals (7). In addition, this study additionally emphasizes the importance of regulation, by intracellular mediators such as cAMP, of immune reactive cells, and in particular their ability to respond in

the production of immune injury. Consideration of such factors may prove useful in future clinical studies of environmental lung disease, and may facilitate further definition of risk factors both at the individual as well as environmental level.

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DISCUSSION

DR. NETTESHEIM: I have two questions. Would you say a word about how you explain the difference between the intra-tracheal delivery and the inhalation delivery and the presence in the serum? Is that, perhaps, related to the amount of BSA delivered to the lung, or is there something else? Whatever happened to the experimental asthma model in guinea pigs, where you immunize with BSA, and then with inhalation of the same antigen; it was very severe asthma. How would that type of reaction fit in with what you have described here?

DR. WILLOUGHBY: It's very difficult to achieve an exact comparison between the two. We get 100 milligrams of BSA, of which 4 milligrams goes in the rabbit. In our instillation, we instill 5 or 10 milligrams. Perhaps the single most important aspect is that when you instill it, it all goes into one small area. We know by counting different lobes in our preparation, it's very profusely distributed throughout the lung. We feel that both are meaningful models. Instillation certainly relates to aspiration, I would think, inhalation perhaps more to the environmental dust situation. Your second question is: The asthma models are airway models, and they're classically carried out in the guinea pig. We're looking at chronic disease. The rabbit works best, because it doesn't have a receptor, and you don't kill the animal. You can kill the guinea pig, unless you work it just right.

DR. WASSERMAN: Do your animals have an immediate hypersensitivity skin test of IgE antibody, BSA? You didn't show us electron micrographs of the Con A-induced lesion alone. Does that look like the interstitial process that you see in Con A plus antigen, or is it somewhat different?

DR. WILLOUGHBY: As far as the IgE response, it was a very tiny response, we have not actually looked by PCA. However, most people feel that Arthus requires microprecipitable antibody for its initiation. By the very fact that we can get an Arthus reaction suggests that with BSA there must be some homocytotropic antibody. And more importantly, since the cholera toxin didn't block it, the cholera toxin must not be having a major effect on mast cells, although it's neutral in vitro. Your second question was about the EM. These are preliminary photographs, and we haven't done the others. It's a reasonable thing to look at, and we probably will.

DR. BASEMAN: How do you account for the half-life of BSA in your model?

DR. WILLOUGHBY: First of all, in the instillation, these animals are not immunized. Secondly, you're looking at a dynamic thing. When you look at the half-life, you inject i.d., and you follow the half-life as it disappears, but it's disappearing in one route, and it's being absorbed from the gut at the same time. We do not see a normal first order decay of the BSA or BSA label in the circulation for several reasons. First and foremost, it's not intact BSA, but has been extensively degraded. Secondly, unlike in most half-life studies, labeled protein, such as BSA, one injected over a short period of time into the bloodstream. In the experiments being considered here, two processes are involved. One is the half-life of the material in circulation, and the second is continuing absorption or reappearance or appearance of new material into the circulation. What we're looking at is a result of two different processes, that is, the appearance of the label in the serum as well as its disappearance. Hence, one does not see a simple first order decay in concentrations. Third, most of these half-life studies are carried over a period of weeks to months. And I don't know exactly what the half-life of the albumin is in the nonimmunized animal, but I suspect it's anywhere from two weeks to four weeks. However, the studies that we're showing are just carried out over a matter of 24 hours. Hence, even if we did classic half-life studies, the line would appear very flat, and one would not see a notable decrease due to catabolism alone.

Lymphatic Removal of Fluids and Particles in the Mammalian Lung

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Physiologists have long realized the importance of the lymphatic vascular system in the removal of fluids, proteins, particulate components, and cells that are not reabsorbed at the venular limb of the blood vascular system (20). Likewise, information from a number of studies has established that the lymphatic system serves primarily as a transport system designed to maintain homeostasis of the interstitial environment by draining excess fluids and proteins from the interstitium for its return to the blood stream (1, 5, 6, 9, 20, 31). The importance of the lymphatic system lies in the fact that even normal blood capillaries are permeable to macromolecules in proportion to their molecular size and that these molecules, particularly proteins, disrupt the normal Starling pattern of exchange between capillaries and tissues. If left undisturbed, this would lead to a considerable accumulation of fluid within the interstitial spaces, leading to edema and altering the hemodynamics of tissue fluids (20). By cannulating the large lymphatic trunks which drain various regions, much information has been obtained regarding the overall contribution of lymphatics, not only in the maintenance of fluid homeostasis for the various tissues throughout the body (4, 5, 7, 21), but also in regard to its role in the removal of hormones (12), enzymes (3, 8, 19, 26), lipoproteins (31), as well as cells (10, 24).

Although we now have some ideas regarding the overall contribution of the lymphatics to the maintenance of fluid homeostasis in various tissues of the body, the precise topographical organization and ultrastructure of this drainage vascular system within the lung still remain unclear.

Notwithstanding the voluminous literature on the anatomy and pathology of pulmonary lymphatics, there have been few definitive studies at the ultrastructural level which consider: (a) their precise topographical arrangement and the ultrastructural basis of interstitial fluid and particulate removal, (b) the structural basis for lymph formation in the lung and the subsequent propulsion of lymph in a unidirectional stream toward collecting vessels, and (c) their participation in pulmonary defense mechanisms and involvement in the genesis and dissemination of various respiratory disease processes including the metastasis of lung cancer.

Just as there is a rich plexus of lymphatic vessels distributed throughout other areas of the body in which there is an abundant amount of connective tissue, the same is true for the lungs (23). In our efforts to gain a new perspective on the old problem of the structural organization of pulmonary lymphatics and their role in the removal of excess interstitial components, we have employed improved techniques of tissue preservation and combined transmission and scanning electron microscopy to study the topography and ultrastructure of pulmonary lymphatic vessels.

MATERIALS AND METHODS

The lungs used in the study were from rats, mice, dogs, and guinea pigs. However, most of the illustrations in this presentation are from the lungs of rats. In an attempt to determine the fine structure of pulmonary lymphatics and their precise distribution within the pulmonary interstitial areas and their close proximity to the alveoli, use was made of combined intratracheal and vascular perfusion methods of fixation.

Surgical Procedure

For control animals, rats were anesthetized with an intravenous injection of sodium pentobarbital. The heart was exposed through a median sternotomy incision and heparin was injected intravenously. Saline containing heparin and procaine were perfused through the lung by inserting a number 260 polyethylene tubing into the pulmonary artery through the right ventricle. The lungs immediately blanched as the saline displaced the blood in the pulmonary blood vessels. After 2-5 minutes, the saline was replaced with a fixative consisting of a glutaraldehyde-formalin mixture (11) or 2.5% glutaraldehyde in phosphate buffer. At the same time as fixative was being perfused into the blood vessels a number 29 gauge needle was inserted into the trachea and the airway perfused with fixative. After the lungs were perfused with fixatives for 15-30 minutes, the hardened lungs were resected and placed in fixatives for an additional two hours. The lungs were cut into small pieces and subsequently processed for transmission and scanning electron microscopy.

Experiments with Colloidal Ferritin and Carbon

To follow the movement of intratracheally instilled particles in the lungs of rats, suspensions of colloidal ferritin (~ 80 Å diameter) or colloidal carbon (~ 350 Å diameter) were administered via the trachea. After periods of 5, 15, and 30 minutes and 1, 3, 6, and 24 hours, the lungs were perfused as discussed above and small pieces processed for transmission electron microscopy.

Dextran Experiments

Dextrans of varying molecular weights (60,000 to 300,000) were injected via the saphenous veins of young adult rats. At 5, 15, 30, and 60 minutes, the lungs were perfused with saline followed by glutaraldehyde-formalin mixture in phosphate buffer at pH 7.4 and at 0°C (27).

Preparation of Lungs for Scanning Electron Microscopy

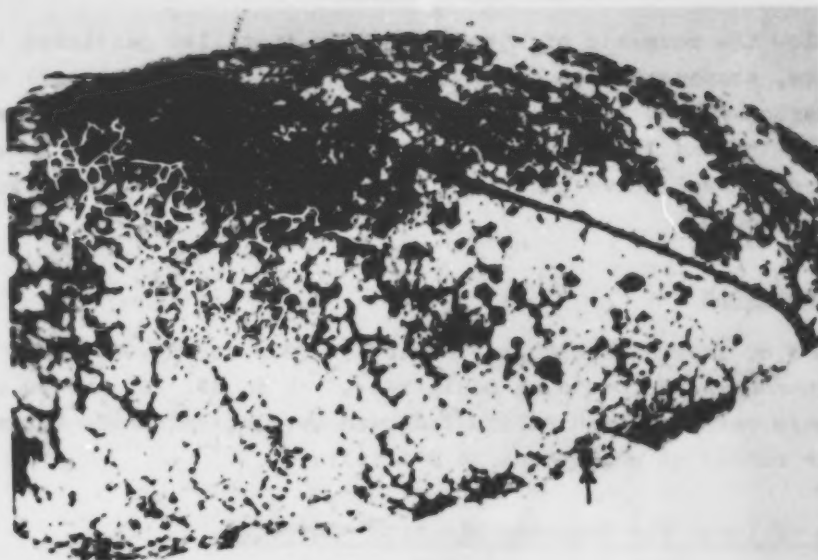
Following perfusion fixation, lungs were cut into small sections and postfixed in 1% OsO_4 at 5°C for one hour, rinsed, and dehydrated in a graded series of alcohols to 100% and gradually infiltrated with Freon 113. The specimens were then processed by the critical point drying method (2, 25) using Freon 13 and a Bomar critical point drying machine. After drying, the specimens were mounted on stainless steel studs with silver conducting paint and subsequently coated with a thin layer of carbon followed by a coating of gold palladium in a Hummer coating machine. Specimens were observed in an ETEC-Auto-Scan scanning electron microscope.

RESULTS AND DISCUSSION

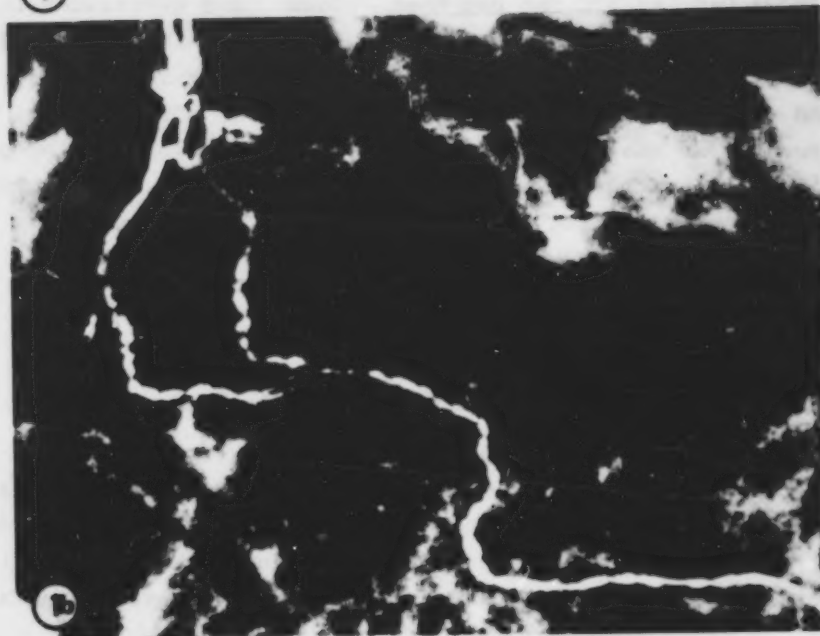
Topography and Ultrastructure of Pulmonary Lymphatics

For a cohesive account of pulmonary lymphatics in relation to their role in the dynamics of lymph formation and the removal of particles and cells from the pulmonary interstitium, it is crucial to give a portrait of the lymphatic vascular system as it relates to the movement of fluids, plasma proteins, and cells across the blood-interstitial-lymphatic interface and the air-interstitial-lymphatic interface in the lung.

In considering the general organization and morphology of pulmonary lymphatics these vessels can be classified by two categories. There is a superficial plexus and a deep plexus of vessels which, unlike the accompanying blood vessels, do not comprise a circulatory system, but a system which subserves the lungs by providing a one-way drainage vascular system for the con-



1a



1b

Figures 1a and b. Radiograph illustrating a rich network of pleural lymphatics in the lower lobe of human lungs. In Figure 1b the periodic constrictions along the length of the vessels indicate the location of valves. (From ref. 30).

stant removal of extravascular fluids, plasma proteins, and cells for their subsequent return to the blood vascular system.

The superficial plexus is located within the connective tissue layer of the visceral pleura. The extensive network of lymphatics within this region is appreciated when these vessels are filled with vital dyes (trypan blue) or radiopaque substances which outline the vessels, making it possible to observe an anastomosing network of vessels (Fig. 1) that contain numerous valves pointing in all directions (23, 29, 30).

The deep plexus of lymphatic vessels is also referred to as the intrapulmonary or parenchymatous lymphatics. It consists of an interconnecting network of vessels which surround bronchi and pulmonary arteries and veins. It is also generally agreed that a rich plexus abounds within the interlobular septa. These vessels are thought to provide interconnections between the deep and superficial lymphatics as indicated by a flow of injected dyes from the pleura to the deep parenchymatous lymphatic plexus (13, 30). Although it is generally agreed that lymphatics abound within the connective tissue sleeves surrounding pulmonary blood vessels and bronchi, the existence of alveolar lymphatics or their presence at the air-blood barrier is still unclear.

By using improved micro-injection techniques combined with vascular perfusion, a rich plexus of lymphatics can be identified within the connective tissue sleeve which extends to the terminal and respiratory bronchioles. These lymphatics are also juxtaposed to alveoli and are separated from the air space only by the alveolar epithelium and a thin layer of adjoining connective tissue. At this level of the bronchial tree, the lymphatics are of a smaller caliber than those surrounding the large bronchi. The lymphatics in these regions are extremely thin-walled and are situated at the sites of fluid formation which, if left to accumulate, would lead to pulmonary edema (13). The smaller and thin-walled lymphatics (10 μ m in diameter) represent the initial segment of the pulmonary lymphatic vascular system, i.e., the lymphatic capillary. Although the continuous layer of endothelial cells is extremely attenuated, many areas between adjacent cells lack adhesion devices. Therefore, adjacent cells are easily separated and can readily accommodate the movement of interstitial fluids and particles into the lymphatic lumen. Therefore, from a functional standpoint pulmonary lymphatics begin as blind end saccules or tubes within a thin band of connective tissue at the level of terminal and respiratory bronchioles.

The unidirectional flow process begins at the tissue-lymph interface with the uptake of interstitial fluids and plasma proteins by the smallest and more permeable vessels, the lymphatic capillaries. From these channels, the lymph is propelled into an extensive system of collecting vessels whose continuity is frequently interrupted by lymph nodes that serve as a filtering or screening system. These vessels contain valves which prevent the regurgitation of lymph.

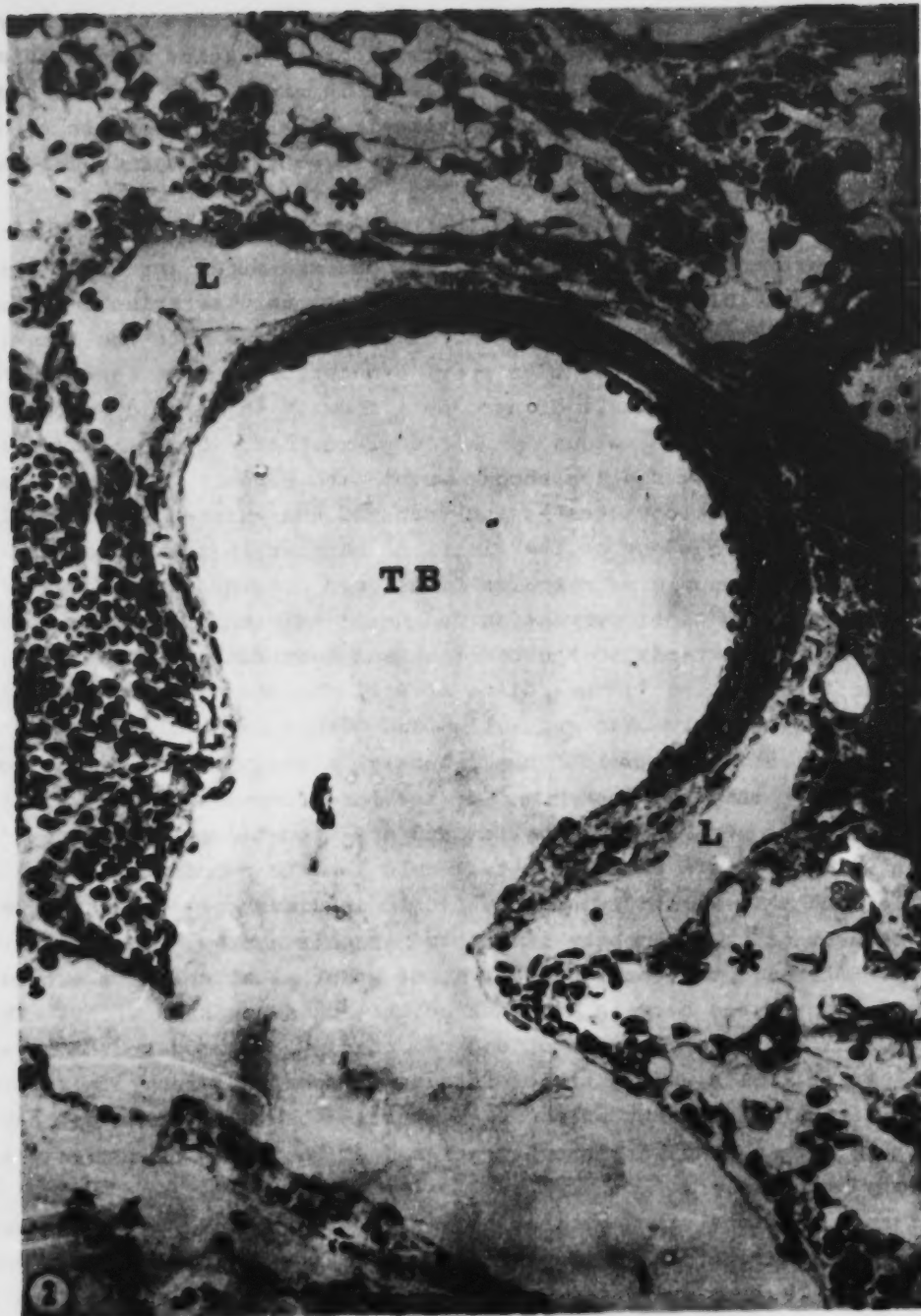


Figure 2. Light micrograph shows the position of lymphatics (L) in relation to a terminal bronchiole (TB) and the adjacent alveoli (*). X900. (From ref. 17).

The collecting vessels usually follow the overall distribution of the arteries and veins within the lung. Lymph drained from the collecting vessels enters the main lymphatic vessels which drain toward the hilar regions of the lungs.

Upon superficial examination of respiratory bronchioles and the adjoining alveoli in paraffin sections, the general organization of the connective tissue is unimpressive. However, on closer inspection of one-micron thick Epon sections, a plexus of lymphatic vessels can be detected at the light microscopic level (Fig. 2). The content of precipitated lymph along with occasional lymphocytes or macrophages make it easier to differentiate lymphatics from blood vessels. Electron micrographs of perfused lung tissue confirm the presence of precipitate within the lymphatic lumen (Fig. 3). In addition, the irregularity of its wall and close topographical relationship to the adjoining alveolar space (sacculi) are also appreciated. In such areas the lymphatic capillary is separated from the air space by the alveolar wall and a thin band of connective tissue. In some areas blood capillaries may be interposed between the lymphatic vessel and alveolar sacculi.

The Ultrastructure of Pulmonary Lymphatic Capillaries

The endothelial cells of the pulmonary lymphatics, like those in other tissues, are extremely attenuated over large areas. At this level of organization, these vessels resemble lymphatic capillaries as no smooth muscle cells were observed in their walls. In addition, they lack a continuous basal lamina and there were numerous anchoring filaments extending into the surrounding connective tissue (Fig. 4). The adjacent cell margins overlap extensively and the intercellular clefts are of variable widths, occasionally accommodating the passage of lymphocytes from the interstitium (Figs. 5 and 6). It is likely that plasma proteins as well as other cells also gain access to the lymphatics in this manner.

Other salient features of the endothelial cells include numerous plasmalemmal invaginations and many microfilaments occur throughout the cytoplasm (Fig. 7). Recent studies of Lauweryns *et al.* (14) demonstrated that the 5-6 nm filaments in pulmonary lymphatic endothelial cells formed the characteristic arrowhead complexes when reacted with heavy meromyosin, suggesting that these filaments represent actin.

Pulmonary Collecting Lymphatic Vessels

Proximal to the terminal bronchiole, the thickness and complexity of the surrounding sleeve of connective tissue are gradually increased. Likewise, the plexus of peribronchiolar and perivascular lymphatics also becomes more elaborate (Figs. 8 and 9). The lymphatic vessels have a larger diameter and a thicker wall consisting of a continuous layer of endothelial cells which are



Figure 3. Survey electron micrograph shows a pulmonary lymphatic capillary (L) and its relationship with a respiratory bronchiole (RB). A gray flocculent precipitate (*) fills the lymphatic lumen and is also located in the connective tissue. The lymphatic capillary is separated from the respiratory bronchiole by a blood capillary (BC) and a band of connective tissue (CT) which contains a thin process from fibroblasts (F) and collagen fibrils (CF). X7,000.



Figure 4. A portion of pulmonary lymphatic capillary illustrating its close relation to the adjoining connective tissue (CT). Numerous anchoring filaments (af) extend from the abluminal surface and project into the surrounding connective tissue. The endothelium (E) contains plasmalemmal invaginations and vesicles (v).



Figures 5a and b. Electron micrographs illustrate the extensive overlapping of adjacent endothelial cells (E) at intercellular junctions (J). The width of the intercellular cleft (*) is also variable. (a) X56,000; (b) X56,000.

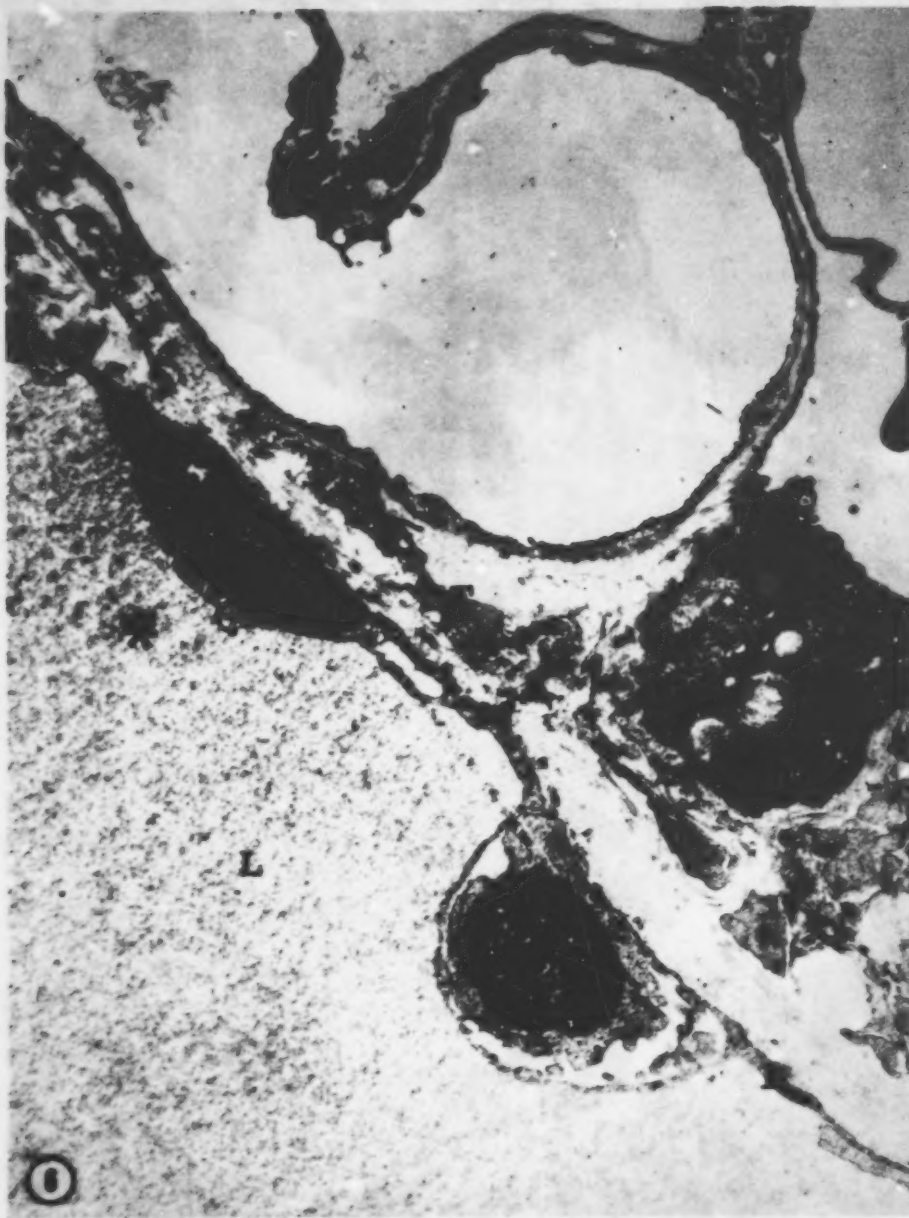


Figure 6. Portion of a juxta-alveolar lymphatic capillary (L) whose lumen is filled with an electron dense precipitate (*). A lymphocyte (ly) is located within the cleft of an intercellular junction (J). The lymphatic endothelium (E) is extremely attenuated except in areas occupied by the nucleus (N). The lymphatic capillary is separated from the alveolar sac by a thin band of connective tissue (CT). X7,600.

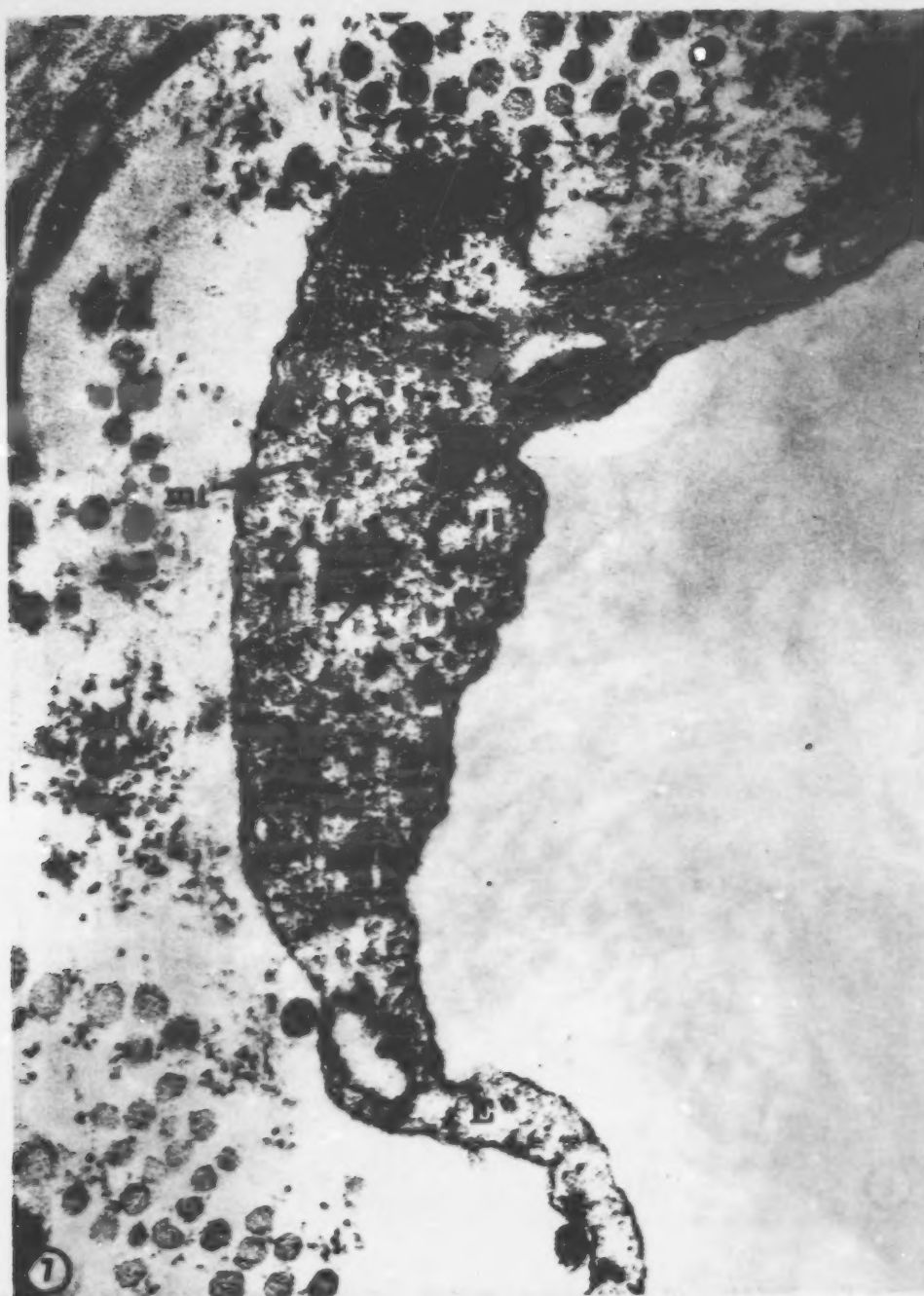


Figure 7. Electron micrograph of a segment of lymphatic capillary endothelium (E) illustrating cytoplasmic filaments (cf), microtubules (mt), and ribosomes (r). X77,700.



Figure 8. Lymphatic vessels within the perivascular connective tissue sleeve (CT) of the larger vessels which still maintain close proximity to the alveolar sac (AS). X7,350.

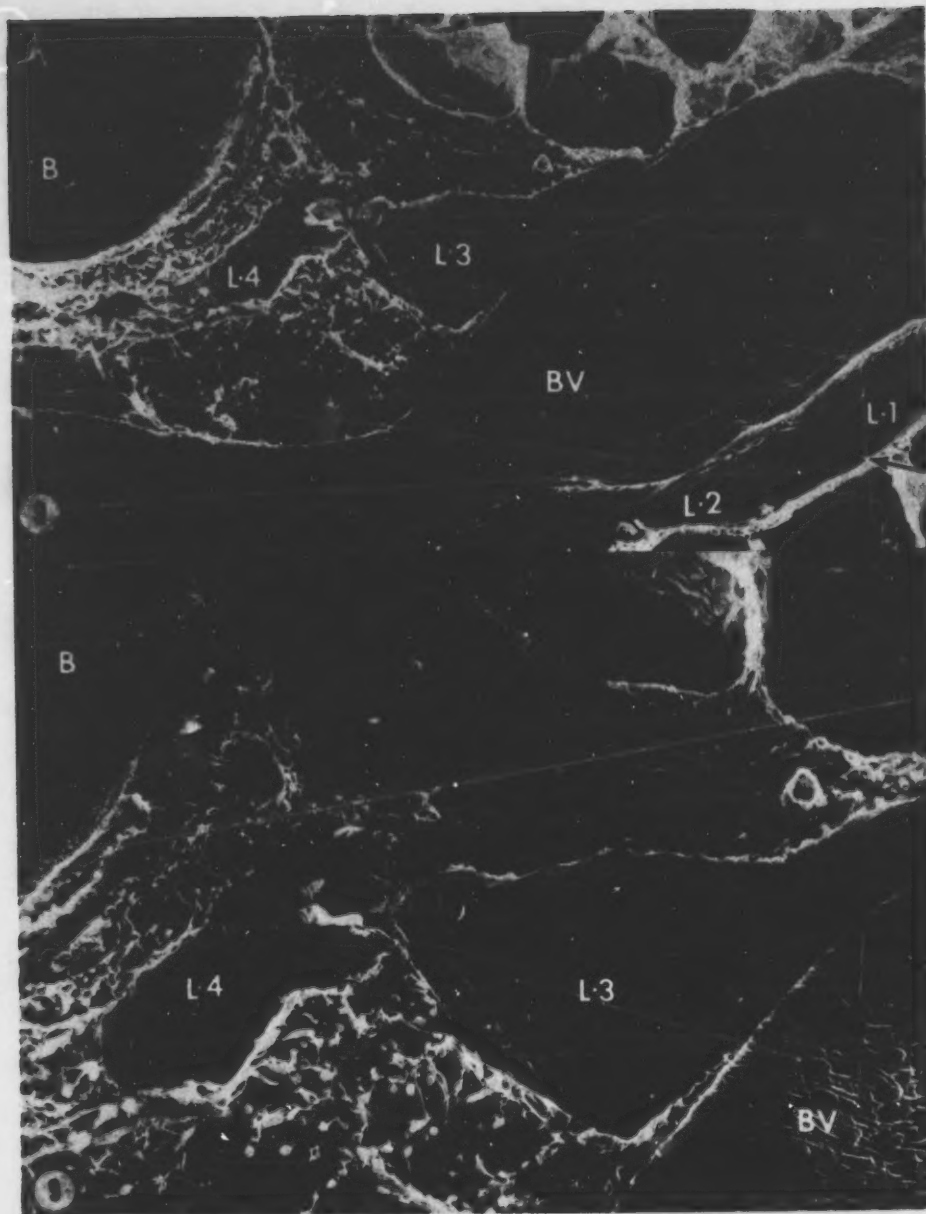


Figure 9. The branching of peribronchial and periarterial vessels is demonstrated in these scanning electron micrographs. In (a) parts of lymphatic vessels are seen at L1 and L2 which contain a clump of cells (arrow). L3 also contains a clump of cells (arrow) and forms an anastomosis with L4 by way of a valve (V). Lymphoid tissue (*), similar to that observed in scanning images of lymph nodes, surrounds the lymphatic vessels. Bronchial (B) and blood vessel (BV) are as marked. (a) X250; (b) X500. (From ref. 17).

held in close apposition by maculae adherents. The wall of the larger vessel is distinguished by the periodic occurrence of valves. The valves consist of leaflets of endothelial cells which project into the lumen as folds (Fig. 10). The two layers of cells are separated by a thin band of connective tissue consisting of collagen and elastic fibrils and an occasional fibroblast (Fig. 10). The regular spacing of valves and constriction at the base of each give a beaded appearance characteristic of the distended lymphatic collecting vessel. When observed in the scanning electron microscope, the bileaflet nature is readily apparent in three-dimensional relief (Figs. 9 and 11). It is evident that the endothelial cells are duplicated as a double layer of cells encircling the lumen of the vessel. The folds are separated along the midline to form paired leaflets which appear as two thin cusps. The surface of each leaflet is lined with flattened endothelial cells. The leaflets project into the lumen at an angle such that their free edges fit together as a miter joint without fusing with each other (Fig. 11). The valves project into the lumen in the direction of fluid flow, providing free movement of lymph toward the larger vessels and lymphatic trunks.

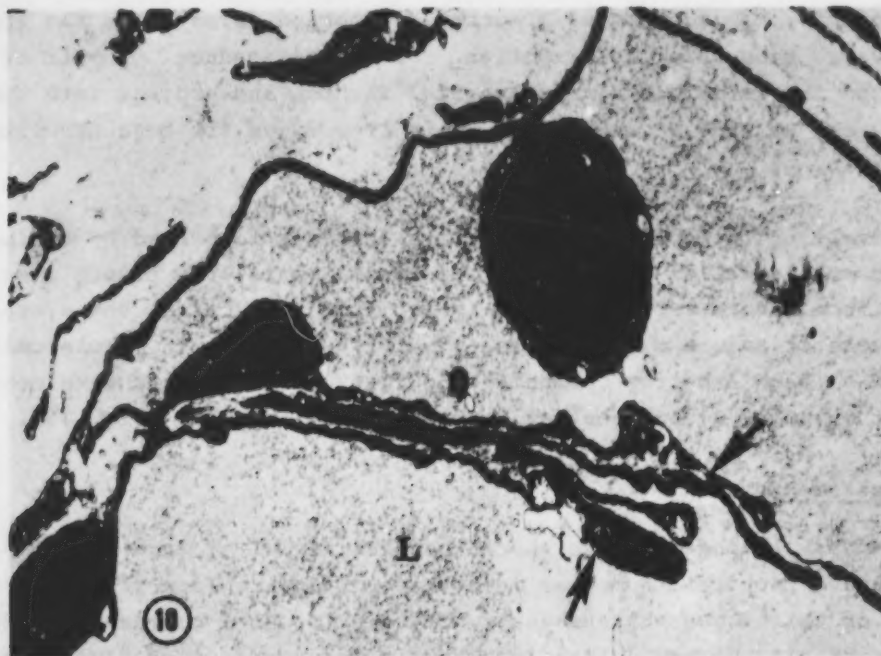


Figure 10. Electron micrograph of collecting vessels illustrating the appearance of valves (arrows) which arise as a duplication of the endothelium which folds into the lymphatic lumen (L). (a) X3,000; (b) X8,000. (From ref. 17).

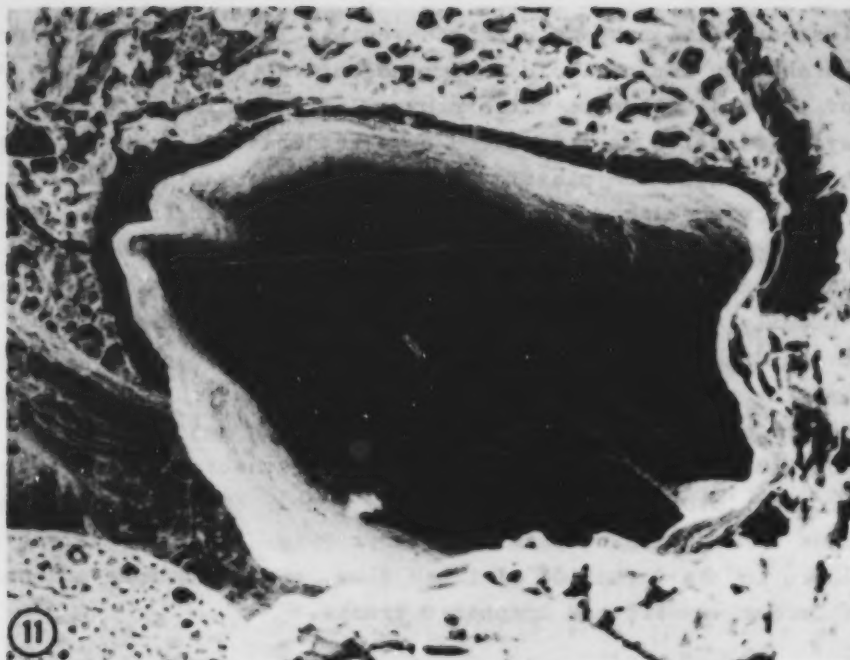


Figure 11. This scanning electron micrograph illustrates the appearance of a valve as seen in a cross-section of a lymphatic duct. A pair of leaflets extend from the wall in a circumferential fashion and project into the lumen of the vessel at such an angle that their free edges fit together like a miter joint. X240.

The wall of the collecting vessel is also distinguished by the presence of smooth muscle cells. Located in the tunica media, the smooth muscle cells may vary from single cells arranged in an incomplete spiral to several complete layers of smooth muscle cells (Fig. 12). The smooth muscle cells are connected to each other by communicating (gap) junctions and make contact with the endothelium by myoendothelial intercellular junctions (Fig. 13).

Trace Experiments

Pulmonary lymphatics also share the distinction of engulfing large molecules and particulate matter from the surrounding interstitium. Advantage is taken of this phagocytic property not only to label pulmonary lymphatics but to ascertain the mechanisms involved in the movement of fluids and large particulate substances across the air-tissue-lymph interface as well as the blood-tissue-lymph interface.

In an attempt to monitor the uptake of colloidal particles from the

alveolar spaces, ferritin and colloidal carbon suspensions were instilled into the trachea of young adult rats. In samples of lungs observed at 15 and 30 minutes after tracer was instilled in the trachea, large amounts of ferritin and carbon could be seen in the lumen of the bronchial tree (Figs. 14 and 15). Tracer particles were also observed in vesicles within alveolar macrophages and squamous cells of the alveolar epithelium (Fig. 16). At time periods of up to three hours, the tracers were observed within vesicles in macrophages located in the interstitium and in vesicles within the lymphatic endothelium (Figs. 17 and 18). For periods of up to six months following intratracheal

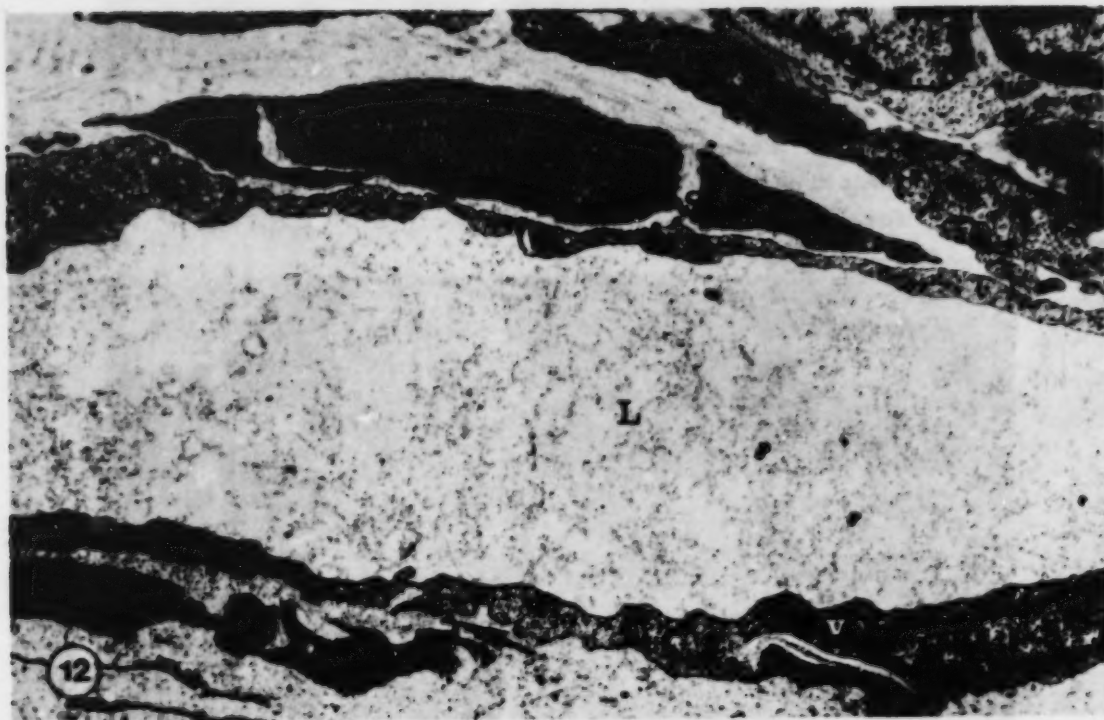


Figure 12. Portion of collecting lymphatic with closely associated smooth muscle cells (SMC), some of which make contact with the endothelial cells (arrows). The lumen (L) of collecting vessel also contains a dense precipitate. The endothelial cells (E) contain the usual complement of organelles including large vesicles (v) with an electron dense substance. X11,000.

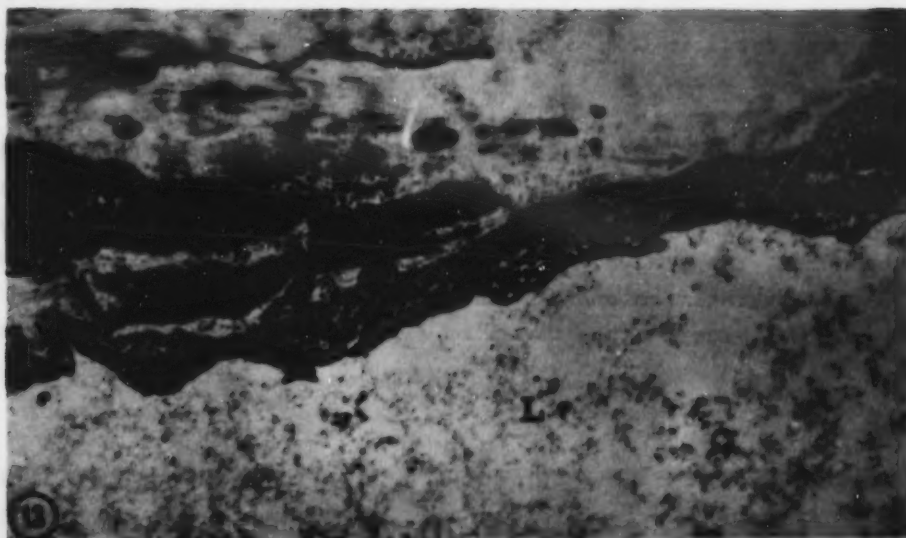


Figure 13. The smooth muscle cells (SMC) of the tunica media and the lymphatic endothelial (E) cells of collecting vessels are closely apposed to each other to form myoendothelial junctions (arrows). X15,048.



Figure 14. An electron micrograph depicting the presence of ferritin particles (*) within alveoli. X20,000. (From ref. 17).

injections of colloidal carbon, the tracer was observed as large accumulations within autophagic vacuoles in the lymphatic endothelium as well as in macrophages located in the lymphatic lumen and in pulmonary lymph nodes. Although ferritin particles were observed free within the interstitium, this was not the case with the carbon tracer observed within vesicles in macrophages and lymphatic endothelium.

Intravascular Injection of Tracer Substances

Although interstitial injections of tracer substances permit the uptake of tracer to be first removed by the lymphatics before their appearance is observed in the blood vascular system, the problem still exists for the development of some trauma due to unphysiological pressure in the vicinity of the injection site, as in the case of interstitial injection, or the production of a mild inflammatory response as indicated by the migration of macrophages and neutrophils to the site of irritation. This possibility is overcome by using intravenous injections of tracer particles or substances which can be rendered electron dense for visualization in the electron



Figure 15. Intratracheally injected carbon is shown within alveolar macrophages. X13,000. (From ref. 17).

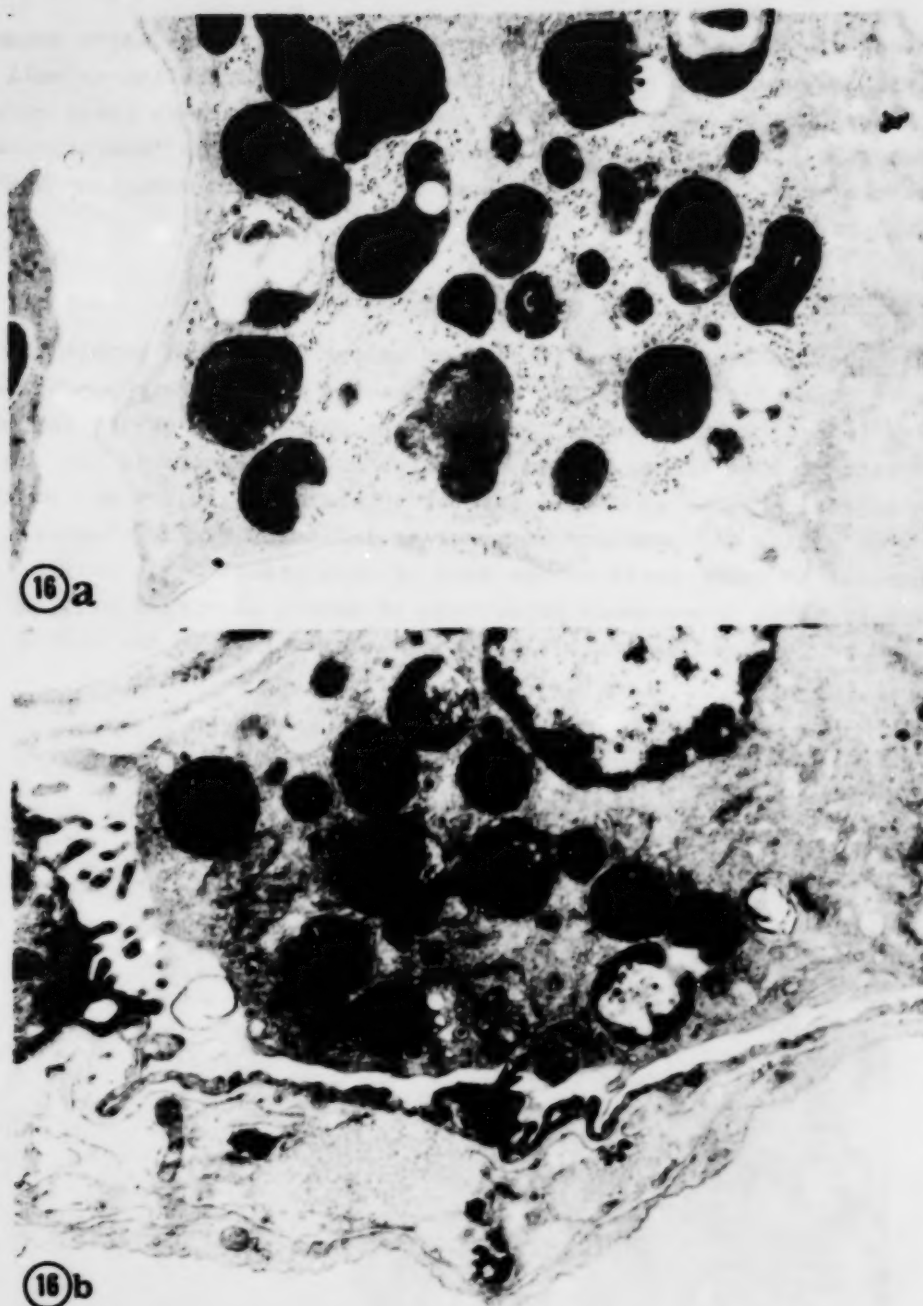


Figure 16. A portion of alveolar macrophage which contains numerous vesicles that are filled with ferritin particles (*). X12,400. In Figure 16b, carbon tracer particles (arrow) are observed in a vesicle within the epithelium lining of the alveolar wall. X15,378.

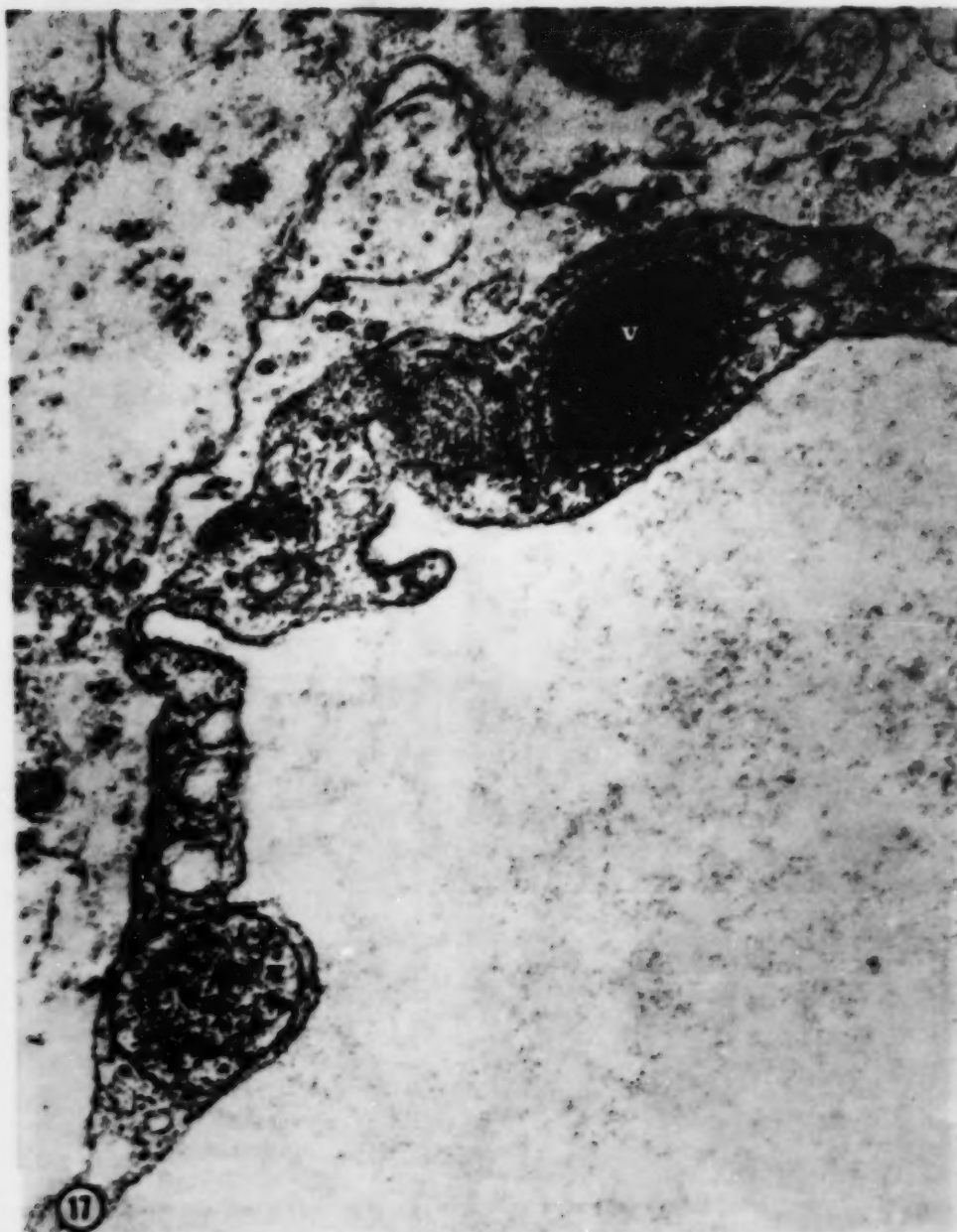
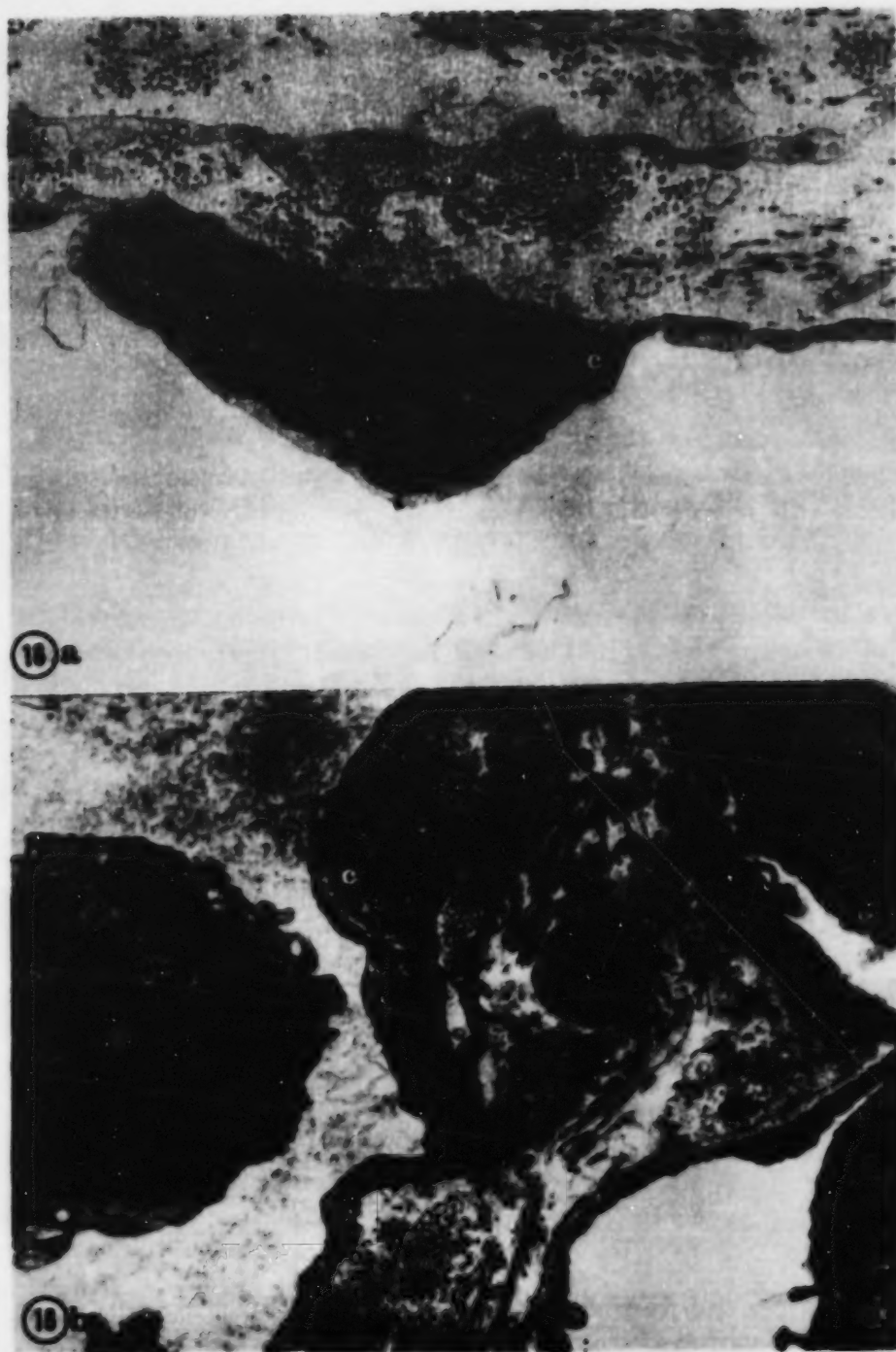


Figure 17. Ferritin is located within large vesicles (v) at 12 hours after injection into the trachea. X79,800.



Figures 18a and b. Carbon particles (C) are also accumulated within vesicles within the lymphatic endothelium (E). (a) X13,224; (b) X10,944.

microscope by chemical means once the tissue has been fixed and processed. This difficulty can be avoided by using the procedures of Simionescu and Palade (27) in which dextran is injected intravenously. Dextrans of varying molecular weights (60,000 to 300,000) were injected via the tail or saphenous veins in young adult rats. It is well documented that dextran injected intravascularly is able to pass across the blood capillary wall and is removed from the interstitium by lymphatic vessels for return to the systemic circulation (9). To observe dextran mainly in the interstitium and the lymphatic vessels, the lungs were perfused with saline to free the blood vessels of injected dextran solutions, leaving the tracer in the interstitial areas and within lymphatics. After fixation with a glutaraldehyde-formaldehyde mixture in phosphate buffer at a pH 7.4 at 0°C (27), dextran particles were retained in a homogenous distribution in the plasma and interstitium, presumably as a result of fixation of the surrounding proteins. This procedure produces an electron dense product whose density is considerably enhanced by postfixation in osmic acid in a phosphate buffer solution.



Figure 19. Part of lymphatic vessel from animal injected with dextran which appears as a very electron dense precipitate (*) in the connective tissue, within vesicles (v) and within the lymphatic lumen (L) at 15 minutes after an injection of a 10% solution of dextran via the saphenous vein. X23,000.

Examination of pulmonary tissue within 15 minutes after dextran injections shows the presence of this tracer throughout the interstitium and within the pulmonary lymphatic vessels (Fig. 19). Dextran gains access to the lymphatic capillary lumen via vesicles and within the intercellular clefts of loosely adherent overlapping intercellular junctions. It is of special interest to note that the uptake of dextran from the surrounding interstitium by pulmonary lymphatics is very similar to the pattern of movement of peroxidase across the blood-tissue-lymph interface in other tissues of the body (16).

The presence of tracer particles within alveolar macrophages and in vesicles of squamous cells of the alveolar epithelium at short intervals (15-30 minutes) and the subsequent appearance of tracer particles in pulmonary lymphatic vessels provide morphological evidence at the ultrastructural level suggesting that large particles are able to cross squamous alveolar epithelial cells within vesicles. Once in the connective tissue compartment, which has a negative interstitial fluid pressure (22), the particles move toward the lymphatics and are subsequently removed by these vessels in a fashion similar to that in other regions of the body. The observation in the present study and those of other workers (13, 28) suggest that particulate materials are able to cross the pulmonary epithelium within vesicles to gain entrance into the pulmonary interstitium. This removal of fluids and proteins from the pulmonary interstitium is similar to the pattern of lymphatic drainage in other tissues (15, 16, 18). Therefore, fluids, particles, and cells are removed from the pulmonary interstitium by way of vesicles and the clefts of intercellular junctions.

ACKNOWLEDGMENTS

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DISCUSSION

DR. BRODY: Do you have any evidence of whether particles that move from the air space by way of the epithelium and reach the basement membrane? Do you have any evidence that those particles reach the small lymphatic capillaries by the basement membrane pathway, before they get to the interstitium?

DR. LEAK: The passage of particles from the air space into the pulmonary lymphatics occurs by (a) endocytosis of particles by the pulmonary epithelium; (b) movement of vesicles across the epithelium and exocytosis of particles onto the tissue front across the basal lamina and into the interstitium; and (c) once in the interstitium particles move toward the lymphatics and enter the lymphatic capillaries via clefts of loosely adhering intercellular junctions and also via plasmalemmal vessels. In our studies which follow the movement of ferritin from the air space into the interstitium we have observed ferritin particles within vesicles of the epithelium, within the interstitium and within vesicles of the lymphatic endothelium and finally within the lumen of the lymphatic vessel. In similar studies with colloidal carbon very few particles were seen free in the interstitium, but most appeared in macrophages within the interstitium.

DR. BRODY: Do you have any evidence that macrophages lift particles and move from the interstitium to the lymphatic space? You show the particles in the lymphatic lining cells and in macrophages in the lymphatics, and one would conclude that they do, in fact, move.

DR. LEAK: We have not been fortunate enough to see macrophages in the process of passing along the clefts of intercellular junctions as we saw for the lymphocytes as illustrated in this study.

DR. BRODY: Can you say that the particles may be picked up in the interstitium by macrophages?

DR. LEAK: Probably so, based on our earlier studies of dermal lymphatics we did observe interstitially injected particles within macrophages which subsequently entered dermal lymphatics via the clefts of intercellular junctions.

DR. BRODY: Do you think that's because it happens so rapidly?

DR. LEAK: Possibly, since the uptake of interstitially injected dye by lymphatic capillaries occurs with surprising rapidity and since lymphatics are more difficult to locate than blood vessels, it is easy to miss macrophages that may be in the process of entering lymphatic capillaries by way of the intercellular junction.

DR. BROMBERG: You've shown us these tight endothelial junctions in the lymphatic capillaries. As you go to bigger lymphatic vessels that have more of a wall, is there a change in the nature of the intercellular junctions? Is there something like a tight junction, there?

DR. LEAK: Yes, there are also tight (occluden) junctions in the lymphatic collecting vessels; however, they do not form a continuous seal or belt (zonule) that is found in epithelial cells. They appear as discrete junctions to form a spot-weld effect. There are two major differences between intercellular junctions in lymphatic capillary and lymphatic collecting vessels. In the lymphatic capillary, the adjacent cells are extensively overlapped, while very little overlapping occurs between adjacent cells of the collecting vessels. The extensively overlapping cells of the lymphatic capillaries are easily separated to form open (patent) intercellular junctions, thus allowing the rapid movement of fluids and particles from the interstitium directly into the lymphatic capillary lumen.

DR. BROMBERG: I was wondering whether those collecting lymphatics actually might have the ability to concentrate macromolecules that they picked up in the interstitial area, the lymphatic capillary area, and move along? Is it possible there are fluid transport mechanisms that, presumably, would require some tight junctions of the epithelium?

DR. LEAK: We have not detected any differences in the electron density of lymph protein particles within the lumina of lymphatic capillaries and collecting lymphatic vessels. It has been suggested by some investigators that there is a mechanism for concentrating lymph within the vessels by a constant removal of water and electrolytes as lymph is propelled up stream. There are, however, no data to support this.

DR. DAVIS: Do you have any evidence to suggest how furosemide has its remarkable effect on pulmonary edema, and does it affect the pulmonary lymphatics?

DR. LEAK: We have not studied the effects of furosemide on pulmonary lymphatics, although we have noticed an increased delatation of lymphatics during pulmonary edema. Lymphatic capillaries can rapidly dilate because the extensively overlapping intercellular junctions are easily separated.

Session III:
Respiratory Tract Cilia

Structural Basis of Ciliary Movement

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All motile somatic cilia, including those of the human respiratory tract, are similar in ultrastructure in that they consist of an axoneme of 9+2 microtubules, surrounded by a specialized extension of the cell membrane. The nine peripheral microtubules of the axoneme differ from other cytoplasmic microtubules in that they are doublets, composed of a complete microtubule, subfiber A, to which is attached an incomplete, shorter set of protofilaments, subfiber B. Important axonemal interconnections extend from specific points on each subfiber A. They are: (a) the two rows of arms, (b) the radially directed spokes, and (c) circumferentially directed interdoublet links. Together these elements function as the ciliary motor, powered by ATP hydrolysis.

In the last decade or so, considerable advances in our understanding of the precise arrangements of these axonemal structures, together with some important analyses of their biochemistry and function, have enabled us to put together a reasonable working hypothesis of the coupling of structure and function within a cilium to produce movement. Most of the basic work on which this account is largely constructed has been derived from studies of systems other than the mammalian respiratory tract. Indeed, mammalian somatic cilia are relatively poorly studied in terms of motile mechanism and basic beat properties (see ref. 9 for earlier review). Although the ciliated cells of the tracheal epithelium do not form a continuous sheet and the exact ciliary stroke of tracheal cilia is not yet understood, it is

evident that these cilia are capable of moving mucus sheets in a coordinated manner to effect respiratory clearance. There is reasonable circumstantial evidence, which will be summarized here, to conclude that the basic mechanism of ciliary motion, like the ultrastructure, is similar in all 9+2 cilia, including human tracheal cilia. It remains to be seen whether the fundamental biochemical details are identical or whether subtle but vital differences will crop up, affecting beat form and cellular control of motion. Additionally, cilia which primarily move mucus may differ from cilia which move water.

Although the basic ultrastructure throughout the length of the axoneme is identical, for example, in sea urchin sperm tails, Tetrahymena or mussel gill cilia, and tracheal cilia, differences do occur at either end so that the origins, overall lengths, and attachments of the individual doublet microtubules are not the same. For example, a sperm tail may be 50 μ m long, a tracheal cilium only one-tenth to one-fifth of that length; one cilium may taper, another have a blunt, rounded end, and so on. One comparison is shown in Figure 1. These differences again are thought to modify the actual form of beat, while the basic motile mechanisms remain reasonably constant.

SLIDING MICROTUBULES IN CILIARY MOTION

Ciliary motion is based on the sliding of the doublet axonemal microtubules. Several major aspects of this sliding microtubule mechanism are now well established:

(1) The axoneme alone is responsible for ciliary motility. Detergent-treated models of cilia beat normally in appropriate solutions containing mM concentrations of Mg^{2+} and ATP. Detergent treatment destroys the integrity of the ciliary membrane, but causes no substantial change in the arrangement of axonemal components. In the intact cell, the ionic environment of the axoneme and concentrations of ATP and divalent cations are maintained and regulated by the ciliary membrane. The presence of the membrane permits cellular control of axonemal function.

(2) The doublet microtubules of the axoneme slide relative to one another without measurable contraction (cf. ref. 9) during bend production in a ciliary stroke. In a recent test of the geometrical predictions of this statement, Shingyoji et al. (13) pipetted ATP onto local regions of Triton-extracted sea urchin sperm whose heads had been attached to a micropipette. This induced local equal and opposite bends of the axoneme, without change in head or tail position, which conforms exactly to the necessary constraints of a sliding system. Further, Triton-treated axonemes exposed briefly to trypsin, then washed and placed in ATP, do not beat but instead disintegrate by telescoping apart (17). Here sliding of individual

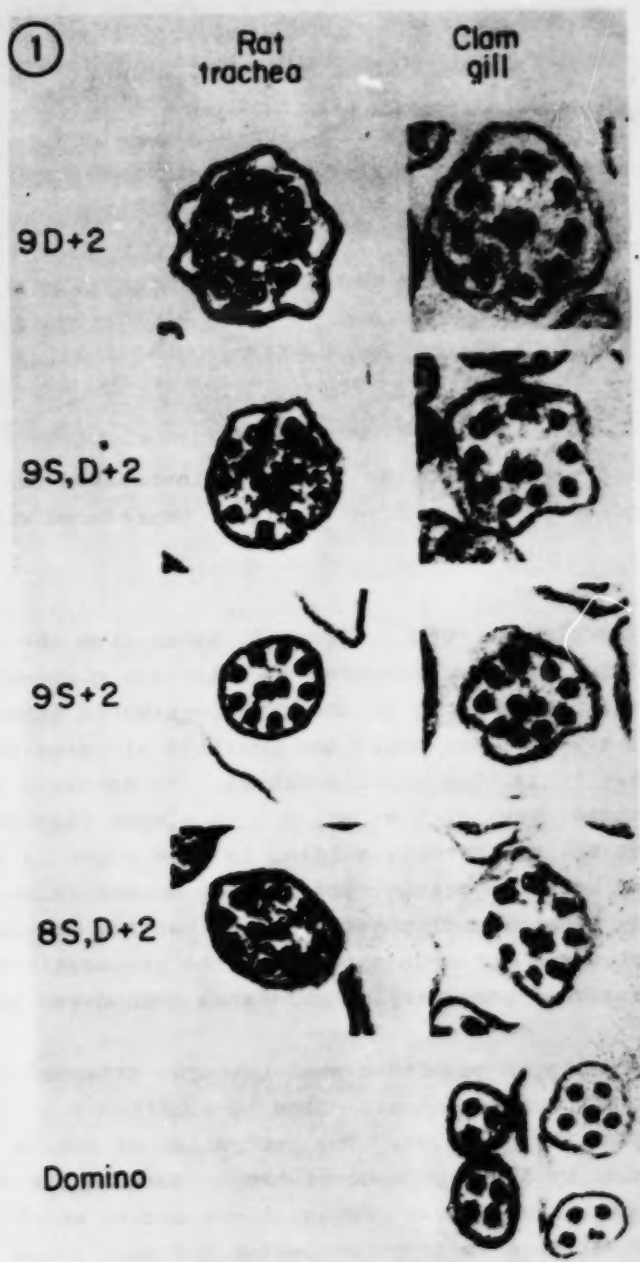


Figure 1. Comparison of ultrastructure of the axoneme (9D+2) and tip configurations of mammalian respiratory and molluscan gill cilia. The finding of 9S+2 and 8S, D+2 tips in separate cilia is indicative of relative sliding of doublets. See ref. 8 for details of nomenclature and interpretation. The tracheal cilia end in a rounded tip and the doublet microtubules are all roughly of equal length; this is not so for the gill cilia, whose tapering tip results in the domino cross-section. X92,500.

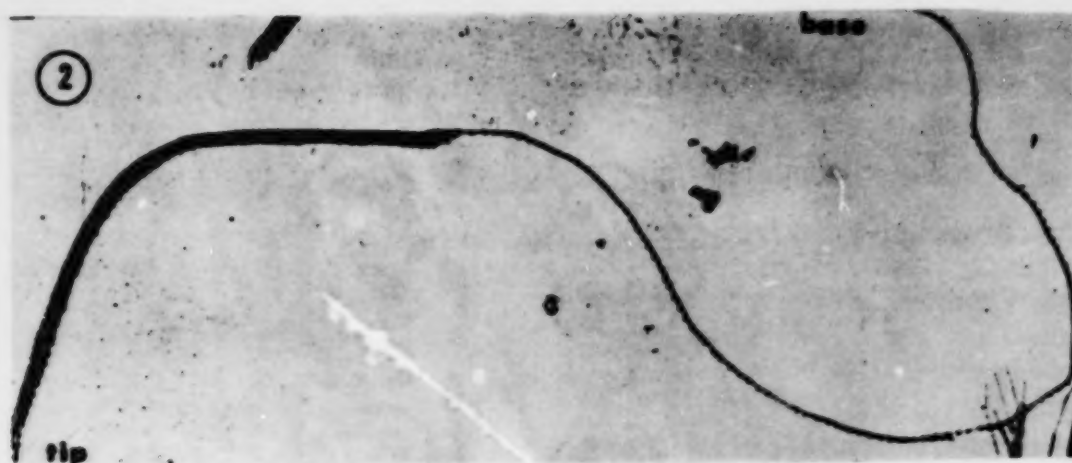


Figure 2. Sliding microtubules in a trypsin-treated ciliary axoneme to which ATP is added for preparative details. Reproduced with permission (12). X18,800.

microtubules is directly observed. Figure 2, taken from the work of Satir and Sale (12), shows an axoneme prepared for electron microscopy after such sliding has taken place. Sliding in the trypsin-treated axonemes is relatively isotropic i.e., at least seven and possibly all nine doublets are capable of sliding. It is also unidirectional. If doublets are numbered 1-9 in the conventional way, doublet no. $n+1$ is always displaced tipward relative to doublet no. n . Lastly, sliding in such cases is uncoupled from bend generation and bend propagation and largely unconstrained: final extension can be many tens of micrometers longer than the original axoneme. Only one constraint seems to limit sliding in the preparations: some overlap of doublets is always necessary, for reasons considered in the next paragraph.

(3) Active sliding is generated when the arms attached to subfiber A of each doublet (i.e., doublet n) also bind to subfiber B of the adjacent doublet ($n+1$) to push that doublet. The generation of active force in this system is accompanied by ATP dephosphorylation. Because the arm action is repetitive and cooperative, we can define an arm cycle, which is discussed below. Presumably, sliding in trypsin-treated axonemes stops when the number of active arms is no longer sufficient to move the doublet microtubules. At that time, evidently, a small amount of overlap between adjacent doublets still remains (Fig. 2). Individual doublets appear inactive when completely separated from the axoneme.

(4) Active sliding and bending are two separable systems; the latter relies on coordination and restriction of sliding, and is trypsin-sensitive.

In the intact (non-trypsin treated) axoneme, microtubule sliding rarely exceeds 0.3-0.4 μ m maximum displacement, that is, only a few percent of total possible sliding. Further, sliding is not unidirectional. This has been interpreted to mean that during beat, doublets slide asynchronously such that the active sliding of doublets no. 1-4 produces the ciliary effective stroke, while doublets 6-9 are passively moved and, alternatively, active sliding of doublets no. 6-9 produces the recovery stroke, while doublets 1-4 are passively moved (12).

Much less is known about the conversion of active sliding into bending and about mechanisms of bend propagation than about sliding itself. The radial spokes, the interdoublet links, and perhaps the arms themselves presumably form part of an elaborate mechanical feedback system within the axoneme that switches sliding on and off appropriately. The spokes, in particular, interact with central sheath projections in a highly circumscribed manner such that as bends develop at the base of a straight axoneme during a beat, the spokes become tilted in a manner consistent with attachment of the spoke head as a method of constraining further sliding and producing bending (22). It seems likely that the spoke-central sheath interaction is central to the local conversion of sliding to bending, or at least to the trypsin-sensitive system which causes the conversion. Spokes, especially the spoke heads, and interdoublet links appear to be the principal structures digested by trypsin under conditions where sliding restraints disappear. Further, mutants of motile Chlamydomonas, lacking either structural component of the spoke-central sheath complex, are paralyzed despite the presence of the intact sliding system (23).

(5) Calcium ion concentration surrounding the axoneme provides a usual control of ciliary activity. Cilia beat normally in the presence of axonemal free $\text{Ca}^{2+} < 10^{-7}$ M. In many organisms, alteration of axonemal free Ca^{2+} to greater than 10^{-6} M causes major alteration in ciliary behavior, including complete cessation of motion (20). However, the trypsin-resistant sliding of ciliary axonemes seems insensitive to changes in Ca^{2+} that physiologically account for ciliary arrest (21), so that it seems probable that the site of Ca^{2+} control resides in the trypsin-sensitive feedback system responsible for bend generation and propagation. In organisms where increasing Ca^{2+} does not cause complete arrest, it is the mode of bend generation and propagation that are usually altered, while sliding must obviously continue.

EVIDENCE FOR THESE MECHANISMS IN RESPIRATORY CILIA

Ciliary malfunction has recently been convincingly documented in man (cf. review in ref. 1). Immotile cilia in males result in a syndrome, formerly known as Kartagener's triad, which couples infertility due to

nonmotile sperm to respiratory disease (sinusitis, bronchiectasis) due to nonfunctional respiratory cilia. (The third aspect of the syndrome described by Kartagener, situs inversus, is not always present and its link to ciliary function is still obscure.) The diseases responsible for the syndrome are congenital and affect females as well as males. Several laboratories have now shown ultrastructural defects present in sperm tail axonemes and somatic cilia in patients whose cilia and sperm are functionally nonmotile. In the original cases, the cilia lacked arms (cf. ref. 1). Sturgess et al. (15) described a second variant, where the cilia possess arms but lack radial spokes. From the complexity of ciliary structure and assembly, it is likely that other variants will be found, including those where the ultrastructural defect is not apparent (Herzon, personal communication). Although the biochemistry remains to be worked out, the mutant classes in humans resemble those in lower organisms where the rationale for ciliary paralysis has been defined. Presumably, in armless axonemes the doublets are incapable of sliding. It would be interesting to know whether the doublets of the spokeless human axonemes would slide upon appropriate treatment; presumably, these axonemes are defective in the conversion of sliding into propagated bending. Despite this impressive circumstantial evidence for mammalian cilia, there is as yet no direct evidence regarding the mechanism of motion. However, sliding of axonemal microtubules in mammalian sperm has been demonstrated (6, 16).

In 1967, Spock (14) reported that addition of serum from cystic fibrosis (CF) homozygotes or heterozygotes to excised rabbit ciliated tracheal epithelium, which was beating in a coordinated manner, resulted in "ciliary dyskinesia." Although much controversy has been generated regarding the existence of specific CF factors, and dyskinesia has never been adequately described in a quantitative manner, ciliary beat clearly looks more or less coordinated under different conditions. Bogart et al. (2) report a CF-like dyskinesia when the divalent cationophore A23187 is added to the medium bathing the tracheal epithelium. Can it be that mammalian ciliary axonemes are also responsive to changes in Ca^{2+} and that dyskinesia is the equivalent of ciliary arrest? If this were so, agents such as cigarette smoke, industrial pollutants, and immune complexes, which might alter the cell or ciliary membrane, could all act to produce dyskinesia via a final common pathway involving increased free axonemal Ca^{2+} .

DYNEIN ARM CYCLE

The aspect of the mechanism of ciliary motility that is proving most amenable to analysis at present is the arm cycle that produces microtubule sliding. The biochemistry of the arms is unsettled in detail, but clear in general principle. The arms are multi-protein structures whose main

functional components are a class of ATPase isoenzymes called dyneins (cf. ref. 5). In *Tetrahymena* ciliary axonemes, the ATPase of the arms seems to correspond to that of sea urchin sperm tails, dynein-1 (4). Dynein-1 can be extracted in a form that has latent ATPase activity and sediments at 21S, and this may represent the intact arm that is the moving cross-bridge between adjacent axonemal doublet microtubules and that generates sliding. The 21S particle is apparently composed of three subunits of about 330K daltons and one each of subunits of 126, 95 and 77K daltons. It is not yet known whether the inner and outer rows of arms are exactly identical in structure or composition.

In negative stain, the subunit construction of the arms is visible (7). The arms have an overall tripartite construction; in at least one position they are slightly tilted (ca. 40°) toward the base of the subfiber A to which they are attached (doublet no. n). In the absence of ATP and the presence of 2 mM Mg^{2+} , both inner and outer arms attach to the subfiber B of doublet n+1, forming bridges between doublets as illustrated in Figure 3a. This is known as the rigor position (3). Such rigor bridges can be plasticized by the addition of ATP, as in Figure 3b (see also ref. 24).

In trypsin-treated axonemes, presumably held together largely by rigor arms in the absence of ATP, upon ATP addition, as the arms are plasticized, the axonemes fall apart, with or without concurrent microtubule sliding (21). This step is evidently intrinsic to the dynein-microtubule interaction per se. For example, Takahashi and Tonomura (18) have recombined dynein arms to doublets from which such arms had previously been extracted. In the presence of Mg^{2+} with no ATP, in a state equivalent to rigor, the arms rebind to and decorate either subfiber A or subfiber B. Upon addition of ATP, equivalent to plasticizing the rigor bridge, the arms detach from subfiber B exclusively.

It is postulated that sliding of the doublet microtubules is accomplished by successive detachment-reattachment of the dynein arms in this way, each detachment requiring binding of new ATP, which is then hydrolyzed at some point in the subsequent cycle, providing the energy for eventual directional force generation. There is little experimentation and no general agreement yet on the exact details of the cycle, but one possibility which combines the known structural information with a plausible, though highly speculative, enzymology is shown in Figure 4. The enzymology is simply drawn as a parallel to the work of Taylor and his colleagues (cf. ref. 19) on the course of actin-myosin kinetics. For cilia, it is known that vanadate is a specific inhibitor of dynein-ATPase activity and doublet sliding, but probably not of doublet detachment (Sale, personal communication). This may prove to be an important tool in clarifying the point in the cycle at which ATP is actually hydrolyzed. Eventually, the combination

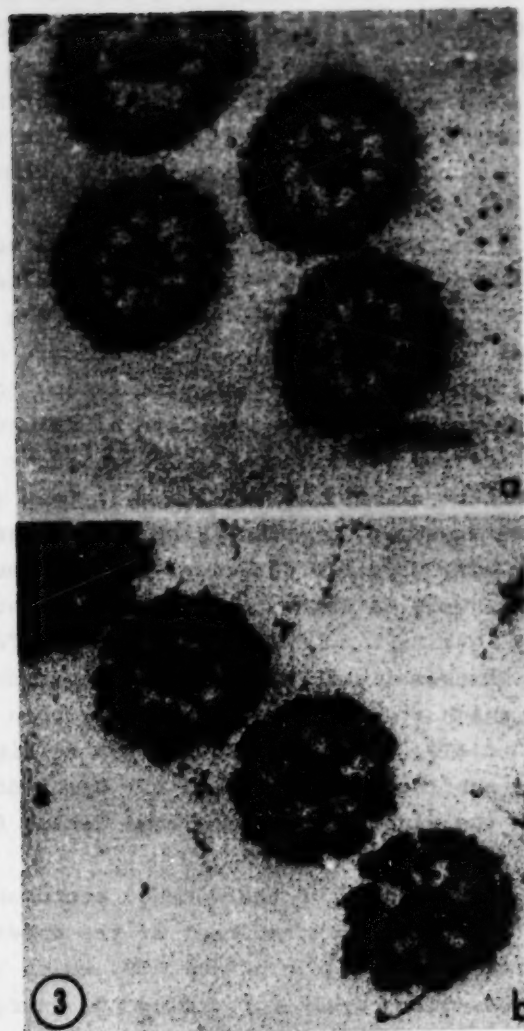


Figure 3. (a) Triton-treated axonemes before ATP addition. All doublets are bridged by rigor arms. X126,000. (b) After ATP addition. Rigor arms are plasticized, so that the doublets are no longer completely attached to one another. X126,000.

of such approaches to the biochemistry, physiology, and morphology of the dynein arm and other axonemal structures should yield a fundamental understanding of the underlying mechanochemical transduction events in ciliary motion.

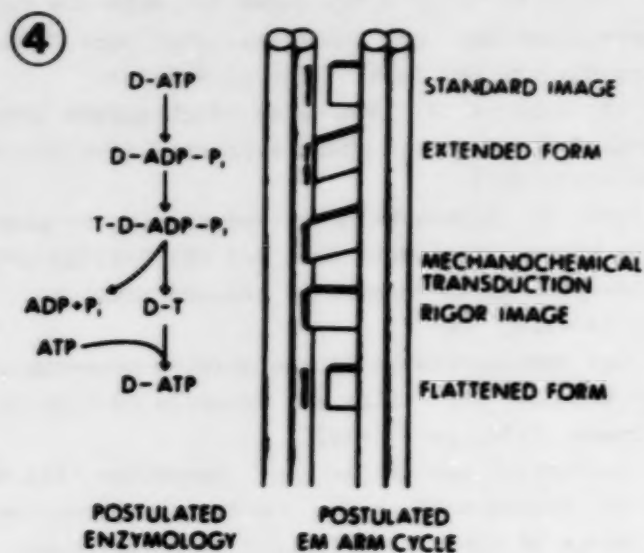


Figure 4. Hypothetical dynein arm cycle (10). From top to bottom successive stages of one complete cycle are shown. At each level, the postulated enzymology is correlated with the postulated arm morphology. The hatched vertical line along subfiber B of doublet $n+1$ as diagrammed indicates hypothetical arm attachment site.

ACKNOWLEDGMENTS

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DISCUSSION

DR. DAVIS: Is there any evidence in the mammalian epithelium that there is a low calcium outside the cell?

DR. SATIR: No, perhaps I didn't make that clear. The calcium outside the cell can be quite high, because presumably of calcium pumps in the ciliary membrane. So, cilia can normally beat in external concentrations of calcium of several millimolar, even though the internal calcium concentration must be often less than 10^{-6} M. In one study, Bogart et al. have used the calcium ionophore. We used the calcium ionophore with gill cilia. They have taken this protocol over to mammalian cilia and have shown that indeed they can reproduce the ciliary dyskinesia which they think they see with cystic fibrosis factors with calcium ionophore. If that's true, then that is a rather important finding.

DR. DAVIS: Is there anything in the cell that would act as a store for calcium, as we have in other cells? In other internal calcium stores that could interact?

DR. SATIR: There are indeed internal calcium stores, and it's thought that cilia can, at least in some cases, respond to nervous stimulation, and this may be in part by releasing internal calcium stores. This still needs to be worked out in detail. I should also say that probably the calcium stores are not from mitochondria. There are other calcium stores in the cell. Mitochondria operate at a different calcium level.

DR. BROMBERG: Dr. Satir, these little beasts are very long and very active; they must be using a lot of ATP per unit length. Does it all get up there from the basal regions of the cell by infective processes, or do you think diffusion is fast enough to account for it?

DR. SATIR: I haven't done the calculations myself, but two or three laboratories have done calculations on the utilization of ATP versus diffusion. They calculate that in the sea urchin sperm tail, which is 50 micrometers long, diffusion is sufficient to supply the appropriate level of ATP. In some cilia there are adenylate kinase and perhaps other enzymes that can generate ATP.

DR. GATZY: Is it my impression that the ciliary beating is sort of a two-dimensional process, even though the shafts are or appear to be fairly uniform?

DR. SATIR: No, that's not right. Ciliary beating is three-dimensional. At least in the gill cilia which I've studied very extensively, one can show that with scanning electron microscopy and by a variety of other techniques. The idea is that there is an effective stroke of the cilium. In the best studied cases, it's clear that this effective stroke of the cilia lies perpendicular to the central microtubules. If you take a line passing between the central microtubules, this is the plane of the effective stroke of the cilium. The cilium then swings out in three dimensions and comes back in a recovery stroke. In fact, it seems that the hydrodynamics of the cilium are such that the cilium is a low Reynolds number system, which means that viscous forces govern motion rather than inertial forces. One consequence of that is the form of beat is important in moving material, and it's important to have a relatively vertical effective stroke and a relatively horizontal recovery stroke.

DR. GATZY: Then you would expect to see specialized structures in the membrane or in the shafts that would be related to localized changes in calcium, since they are likely to occur at some point in the cycle everywhere.

DR. SATIR: We don't know whether calcium governs the normal beat cycle. That is not the case.

DR. GATZY: Or magnesium or whatever?

DR. SATIR: As far as magnesium and ATP are concerned, the cycle looks isotropic, as far as the inside of the axoneme is concerned. In other words, we could take the isolated axoneme, and put it into ATP and magnesium of the appropriate concentrations, and it works. If the membrane simply maintains that concentration around the axoneme, presumably it will work.

DR. GATZY: To get the bending, some areas have to behave passively and some actively. Is that correct?

DR. SATIR: Yes, that's what I said.

DR. GATZY: There must be some localization.

DR. SATIR: That's right, and we don't really understand that.

Injury of Respiratory Epithelium

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The respiratory tract of humans provides a large surface area that is constantly in contact with the environment. The lower respiratory tract is normally sterile, emphasizing the remarkable efficiency of the defense mechanisms of this organ system. At the present time little is known about the effects produced by inhalation of foreign particulates and infectious agents on the epithelial lining of the respiratory tract. It is known that for the respiratory tract to function properly the alveolar macrophages and elements of the mucociliary escalator must be intact. Once these defense mechanisms have been altered, a buildup of both viable and nonviable inhaled substances occurs on the epithelium which may jeopardize the health of the host.

The anatomic and physiologic complexities of the respiratory tract have presented problems in interpreting the effects of exogenous agents on this organ system, since the data base on normal function has not been well delineated. This has led us to establish less complex in vitro respiratory organ culture models that can be more easily observed under controlled conditions. In this paper, studies will be reviewed in which tracheal organ culture has been used to examine the pathogenic potential for epithelial injury by Mycoplasma pneumoniae, Bordetella pertussis, respiratory syncytial virus, and parainfluenza virus type 3. The manner in which these human respiratory pathogens injure respiratory epithelium in tracheal organ culture will be contrasted.

TRACHEAL ORGAN CULTURE MODEL

Tracheas removed from most animals and human fetuses are suitable for study in this model system (3, 4). The organ culture is prepared by removing the trachea with sterile technique. After a midline incision is made in the anterior neck, the trachea is exposed and transected at the levels just below the larynx and above the carina. The trachea is then cut into rings with a scapel. Each ring contains a cartilage which provides support. The rings of tissue are placed on areas of cross-hatch scratches in a small plastic petri dish and enough tissue culture medium added to just cover the rings. The culture is then maintained at 36°C in 5% CO₂ and air.

The inner surfaces of the tracheal rings are lined by organized, differentiated respiratory epithelium that can be maintained up to six weeks when the medium is changed every three days. At any time, the dish may be removed from the incubator and the epithelium examined with an inverted microscope without opening the petri dish. This permits one to examine for the presence and vigor of beating of the cilia which serves as a good indicator of viability of the epithelium and degree of injury produced by test substances.

One trachea provides 13-15 rings, thus permitting tissue with the same genetic makeup, for test and control dishes. This model is very maneuverable, in that rings of tissue may be added or removed from a dish at any time under sterile conditions. The rings can be grasped on their outer surface by forceps and moved without touching their epithelial inner surface.

The animal may be exposed to the test substance prior to sacrifice and removal of the trachea for organ culture, or the tracheal rings may be prepared and then exposed to the test substance. In our laboratory we have had more experience with the latter method.

MYCOPLASMA PNEUMONIAE IN HAMSTER TRACHEAL ORGAN CULTURE

Mycoplasma pneumoniae is the most common cause of pneumonia in teenagers and young adults. This microorganism is the smallest infectious agent capable of growth on lifeless media. In an attempt to better understand the pathogenesis of disease produced by this microorganism, hamster tracheal organ cultures were inoculated with both virulent and attenuated strains of M. pneumoniae and other human mycoplasma species that produce no known disease in man (Mycoplasma fermentans, Mycoplasma hominis, Mycoplasma orale and Mycoplasma salivarium). The virulent strain of M. pneumoniae produced ciliostasis by day 3 and the attenuated strain by day 6. The other strains of mycoplasmas had no effect on ciliary activity, implying that the ciliostatic property possessed by virulent M. pneumoniae might play a role in the virulence of this respiratory pathogen (6).

To be able to quantitate the ciliary beat frequency, a calibrated stroboscope was used as the light source for the inverted microscope (4). The speed of the ciliary beat could be determined from the flash frequency which gave the illusion of arrested ciliary motion. A series of separate cultures was established containing tracheal rings from the same animal; tenfold dilutions of the virulent M. pneumoniae organism pool were employed as inocula. The control rings were placed in sterile broth. Cessation of ciliary activity was produced within four days by the two greatest inocula, while the smallest inoculum required seven days to reach this point. This provides evidence of a dose response effect and suggests that a critical degree of infection is required for tissue injury to occur.

Individual tracheal rings can be removed from the culture dish at any time and examined by microscopy techniques. When tracheal rings fixed with formalin and stained with hematoxylin and eosin were examined, there was good preservation of the ciliated epithelium in the control rings after 48 hours in organ culture. In contrast, the epithelium of comparable tracheal rings infected for 48 hours in organ culture with M. pneumoniae demonstrated nuclear swelling with chromatin margination, cytoplasmic vacuolization, and loss of cilia from some epithelial cells (4, 6).

In an attempt to localize the M. pneumoniae organisms in relationship to the injured cells, tracheal rings were removed from the organ culture and frozen sections prepared. These sections were treated with rabbit M. pneumoniae antiserum followed by goat anti-rabbit globulin conjugated with fluorescein isothiocyanate. When the M. pneumoniae infected tissue was examined with an immunofluorescent microscope, the antigen was found to be located along the luminal border of the epithelial cells. In tissue that had been infected for 72 hours, clumps of specific antigen were seen in the lumen and down into the epithelium on each side of the nonstaining nuclei. Control tissue demonstrated no specific fluorescence. Since the M. pneumoniae were below the resolution limits of the immunofluorescence microscope, it was not possible to delineate their exact location with respect to the epithelial cells by this technique (7).

To answer this question, the tracheal rings were examined by electron microscopy. Tissue was removed from the organ culture dish, fixed in glutaraldehyde and osmium tetroxide, embedded in Epon, and thin sections cut. When tracheal rings infected for 48 hours were examined, densely staining filamentous organisms were seen down between the cilia. At higher magnification the mycoplasma organisms were seen to attach to the epithelial cell by a specialized tip structure. The tip structure consisted of a dense central filament surrounded by a lucent space enveloped by an extension of the organism's unit membrane. The outer membranes of the microorganism and mammalian cell were in close approximation, but were not fused (4, 6).

On further examination, M. pneumoniae were frequently seen attached in the region of the terminal bar where the luminal surfaces of individual epithelial cells were joined. Organisms were also seen penetrating the intercellular spaces, and multiple organisms forming microcolonies were seen in intercellular spaces between epithelial cells (4).

Tracheal rings infected for 72 hours revealed the pathologic changes of both epithelial cell cytoplasmic vacuolization and loss of cilia. M. pneumoniae organisms were seen in the intercellular spaces but no organisms were seen within the epithelial cells. This explained the findings seen with immunofluorescence and the thicker frozen sections. The antigen was present in the intercellular spaces (6).

Another technique that we have used to examine the tracheal organ culture model has been that of scanning electron microscopy (16). This method of observation permits an in-depth look at the luminal surface of the epithelial layer. Numerous filamentous M. pneumoniae organisms were seen attached to the luminal border, and the apical portions of infected ciliated cells were seen sloughed into the lumen. The phenomenon, called ciliocytophthoria, was found to be the characteristic pattern of epithelial cell injury produced by M. pneumoniae infection.

The tracheal organ culture model permits the examination of the biochemical basis of the alterations produced in epithelial cell injury by infectious agents (10-12). Exposure of hamster tracheal rings to virulent M. pneumoniae organisms in organ culture leads to alterations in macromolecular biosynthesis and metabolic activity of the respiratory epithelial cells prior to the occurrence of histopathologic changes. These changes were not seen with avirulent organisms derived from the same parent strain. Within 24 hours after infection of tracheal rings by virulent M. pneumoniae, inhibition of host cell ribonucleic acid and protein synthesis was evident. The addition of erythromycin 24 hours or earlier after infection prevents the onset of abnormal orotic acid uptake and subsequent cytopathology. However, 48 hours after infection, rescue of host cells by erythromycin does not occur and pathological changes become evident.

Our data indicate that the intimate contact between virulent mycoplasmas and the respiratory epithelium alone does not account for the subsequent interruption of host cell metabolism, but must be accompanied by continued multiplication and biochemical function of attached mycoplasmas. The mechanism of injury mediation is not yet known, but the primary effect of mycoplasma infection on tracheal organ culture may be at a transcriptional or translational level.

MYCOPLASMA PNEUMONIAE IN HUMAN FETAL TRACHEAL ORGAN CULTURE

Human fetal trachea in organ culture has also been used to study the epithelial changes produced by M. pneumoniae (2, 5). The ability of mycoplasma species to produce ciliostasis was very similar to that reported above in hamster tracheal organ culture. Only virulent M. pneumoniae injured the human tissue. When examined by light microscopy, the epithelial changes produced by M. pneumoniae followed the attachment of the organisms between the cilia. This was followed by the apical portion of the cell becoming eosinophilic and protruding into the lumen with the cilia forming a sunburst pattern. The ciliated apical portion of the cell was later sloughed into the lumen, with the basal portion of the cell being left behind to maintain the epithelial barrier.

Immunofluorescence studies, as previously described with tissue from the hamster tracheal organ culture system, showed the M. pneumoniae antigens to be localized on the epithelial luminal border between the cilia of the human tissue. The organisms were also seen to invade the submucosal glands.

When the human tracheal tissue was examined by electron microscopy, the M. pneumoniae organisms were seen to attach to the luminal aspect of the cell by their specialized tip structure. No evidence of organisms entering the epithelial cells was seen. The ciliocytophthoria pattern of epithelial cell injury produced by M. pneumoniae was also observed at the electron microscopic level. The luminal portion of the ciliated cell first protruded into the lumen and was followed by a constricting and pinching off of the apical surface. The injury produced by M. pneumoniae may be due to parasitization of individual cells, since heavily parasitized cells showed marked damage of subcellular organelles, and adjacent less heavily parasitized cells appeared normal.

BORDETELLA PERTUSSIS IN HAMSTER TRACHEAL ORGAN CULTURE

The hamster tracheal organ culture model has been employed to examine the effects of B. pertussis on respiratory epithelium (8). This human bacterial pathogen is the causative agent of whooping cough in children. When the tracheal rings were infected with phase I B. pertussis and examined by light microscopy, the bacteria were seen attached to the ciliated cells, but, unlike M. pneumoniae, there was no attachment to the nonciliated cells. After attachment, the ciliated cells were injured and later extruded from the epithelial layer. The extruded cells were seen to contain pyknotic nuclei with bacteria attached only to their luminal surface. After 72 hours of infection in organ culture, no ciliated cells remained, and the spaces left by the extruded ciliated cells in the epithelial layer had been filled in

by the nonciliated cells. No attachment of B. pertussis to the remaining nonciliated cells was observed.

Injury of the ciliated epithelial cells was also examined by electron microscopy. Transmission electron photomicrograph showed ciliated cells that were heavily infected with B. pertussis. Some of these ciliated cells were in the process of being extruded from the epithelial layer. These cells possessed markedly damaged subcellular organelles while the adjoining cells appeared normal. At no time were B. pertussis organisms seen within the epithelial cells.

We have examined comparable tracheal epithelium infected in organ culture with B. pertussis by scanning electron microscopy (15). After 48 hours of infection, multiple extruded cells were seen on the luminal surface. This technique, at higher magnification, also provided a three-dimensional view of parasitized ciliated cells being extruded from the epithelial layer. The ciliated cells that had been extruded had bacteria attached to their luminal surface and there were marked membrane changes, with some appearing as hollow shells with their nucleus having been expelled. No B. pertussis organisms were seen attached to nonciliated cells.

RESPIRATORY SYNCYTIAL VIRUS IN HUMAN FETAL TRACHEAL ORGAN CULTURE

Respiratory syncytial virus is an important cause of bronchiolitis in young children. The response of the respiratory epithelium in organ culture to infection with respiratory syncytial virus is different from that of M. pneumoniae and B. pertussis. When the epithelium was examined by light microscopy, the first changes seen to develop were perinuclear inclusion bodies in the ciliated cells. This was followed by swelling of the ciliated cells with protrusion into the lumen and loss of cilia. Respiratory syncytial virus infection produced fusion of the infected cell membrane with the cell membranes of adjoining epithelial cells, thus forming a giant cell containing multiple nuclei (9).

When human tracheal rings are removed from the organ culture, after infection, and stained with fluorescent antibodies that are specific for the antigens of respiratory syncytial virus, individual cells along the luminal border are seen to fluoresce. The respiratory syncytial virus was found to infect only ciliated cells in the epithelial layer. No specific fluorescence was seen in cells of the lamina propria or cartilage layer.

Tissue from organ cultures comparable to that just described was processed for electron microscopy and examined. Viral particles were seen budding from the luminal aspect of the ciliated cells and from the surface of the cilia. The typical perinuclear inclusion bodies produced by respiratory syncytial virus infection of epithelial cells were seen to be made up of

a dense intertwining of RNA cores. These cores were later packaged at the membrane of the cell where they formed new viral particles.

PARAINFLUENZA VIRUS TYPE 3 IN HAMSTER TRACHEAL ORGAN CULTURE

Another important viral pathogen of children is parainfluenza virus type 3. The epithelial cell injury produced by this virus has been studied in hamster tracheal organ culture (13). Thirteen days after infection in the organ culture the formation of giant cells, similar to those seen in the human epithelial tracheal organ culture with respiratory syncytial virus, was observed. The giant cells of epithelium infected with parainfluenza virus type 3 did not lose their cilia. This virus also produced changes in cells of the lamina propria and cartilage in this system with the formation of multinucleated giant cells. Immunofluorescence microscopy showed parainfluenza virus type 3 specific antigen not only in the epithelial layer but also in the lamina propria and cartilage cells. When comparable tissue was examined by electron microscopy, typical syncytial formation of epithelial cells was observed. Virus particles were observed budding off the cells in the epithelial, lamina propria, and cartilage layers of the trachea.

CONCLUSION

In this paper I compared the changes produced in organized, differentiated epithelium maintained in tracheal organ culture by different infectious agents (M. pneumoniae, B. pertussis, respiratory syncytial virus, and parainfluenza virus type 3). This simple system demonstrated that differentiated, organized epithelium responds differently to individual infectious agents. This model has been useful in defining the susceptible cell type in the organized epithelium of pathogens. Only limited use of this model has been made in studying the effects of pollutants on respiratory epithelium. Some of these are: 1) Adalis et al. have examined the effects of cadmium on epithelium in hamster tracheal organ culture (1). 2) Schiff studied the effects of nitrogen dioxide on influenza virus infection in hamster tracheal organ culture (17). 3) Mossman et al. reported on the effects of carbon particles on tracheal epithelium in organ culture (14).

The in vitro tracheal organ culture model should provide a valuable screening tool to further explore the injury produced by different pollutants and to establish the lowest levels of pollutants required to produce epithelial morphological and metabolic injury. The model should also prove useful to study synergism between infectious agents and pollutants.

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DISCUSSION

DR. ADALIS: Dr. Collier, have you observed changes in the number of ciliated cells and the location of the nuclei within the ciliated cells during organ culture?

DR. COLLIER: The ciliated cells do not replicate and we do see a decrease in this cell type particularly after three weeks in organ culture. I have not observed changes in the location of the nuclei within the ciliated cells.

Session IV:
Model Systems of
Respiratory Infectious Diseases

Interaction Between Environmental Pollutants and Respiratory Infections

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During this morning's session we have heard several papers discussing the mechanisms which are active in protecting the respiratory tract against damage due to inhalation of airborne contaminants. The mechanisms include the mucociliary system, phagocytic activity of alveolar macrophages, and the immunity of the respiratory tract. These mechanisms are indeed the same which play a significant role in protecting a host against respiratory infections. Thus, the effects of air pollutants on the respiratory tract are of special importance in relation to respiratory infections.

Health effects studies of air pollutants have been usually concerned with the causal association between a single pollutant and a disease state. However, it is well known that frequently more than one factor is responsible for the occurrence of natural diseases. Therefore, multiple causalities must be considered in the assessment of biological effects of air pollutants. One such interaction is depicted by the animal model system which reflects the exacerbation of bacterial respiratory infections by inhalation of air pollutants. Indeed, changes in the resistance to respiratory infections provide a highly sensitive experimental model which is with increasing frequency used in studies of health effects of air pollutants.

The relationship between exposure to air pollutants and the resistance to respiratory infections has been investigated in our laboratories since 1955. The gaseous pollutants included in these studies were ozone, nitrogen dioxide, sulfur dioxide, and peroxyacetyl nitrate. Particulate pollutants in-

cluded sulfuric acid, various sulfates and nitrates, and oxides of platinum, palladium, manganese, and nickel. The studies were designed to determine the effects of single and multiple exposures to a single pollutant or to pollutant mixtures.

MATERIALS AND METHODS

The experimental protocol used in the studies calls for exposure of a group of animals, usually 6- to 8-week-old mice, to a pollutant and another group to clean air. After the exposure the two groups of mice are combined and within less than an hour challenged by the respiratory route with an aerosol of the infectious bacteria, either Streptococcus pyogenes or Klebsiella pneumoniae. The challenge usually lasts for 10 to 15 minutes and results in the deposition of approximately 750 to 1500 viable bacteria per lung. After the challenge the mice are kept in an isolated clean-air room for a 14-day observation period. The changes in resistance to the infection are usually measured by two parameters, namely, changes in mortality rates and in survival time when compared to those seen in infected mice exposed to filtered air. In addition to mortality and survival, several other parameters are measured. These include clearance rate of inhaled viable bacteria from the lungs, damage to the respiratory tract tissue as observed by conventional histopathological and scanning electron microscopic examination, development of lung edema, activity and function of alveolar macrophages, humoral and cell-mediated immune responses, and various clinical pathology measurements. To determine the statistical significance of the differences in responses between animals exposed to the pollutants and those exposed to clean air the chi-square analysis, Student's t-test, analysis of variance, and linear regression analysis were used, as appropriate.

The details of experimental methods used for preparation of the environmental atmospheres, monitoring of the pollutants, infectious challenge with the bacterial aerosols, and the assay of the various health parameters were described in previous publications (2-4).

RESULTS

Effects of Gaseous Pollutants

Figure 1 shows mortalities resulting from a single 3-hour exposure of mice to either nitrogen dioxide or ozone and challenge with Streptococcus aerosol (3). Statistically significant increases ($p < 0.05$) in actual mortality are seen after the single exposure to either $3.76 \text{ mg/m}^3 \text{ NO}_2$ or $0.2 \text{ mg/m}^3 \text{ O}_3$. These data indicated the high sensitivity of this assay system, whereby a significant physiological response can be attained at concentrations of air

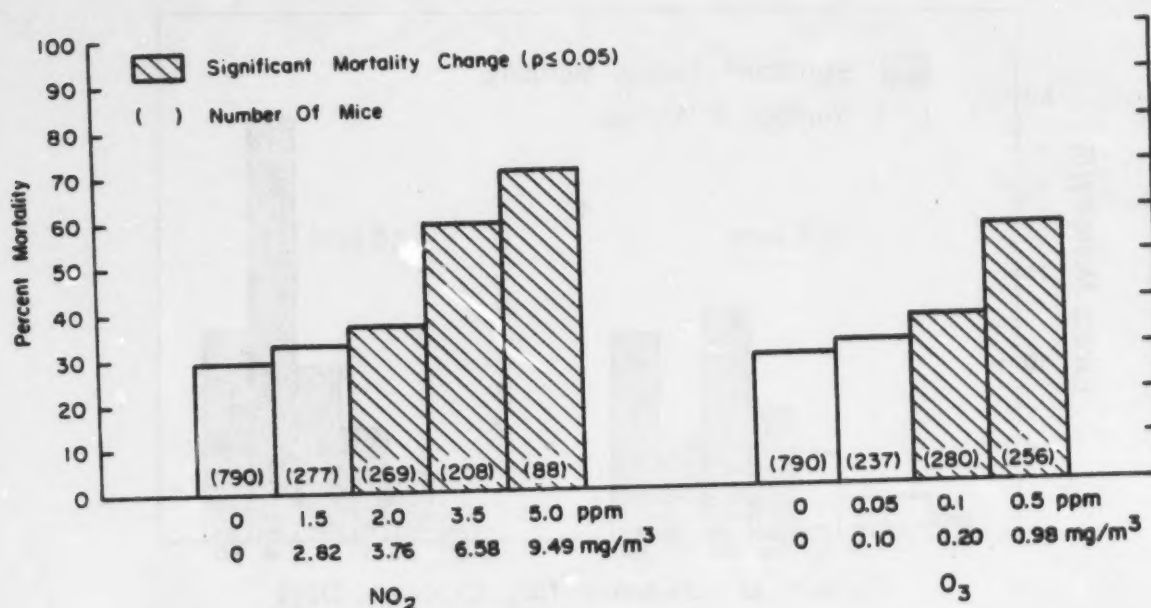


Figure 1. Mortality rates in mice exposed for 3 hours to O₃ or NO₂ and challenged with Streptococcus aerosol (3).

pollutants frequently found in urban environments. The mortality rates of mice exposed to ozone are in a close agreement with those reported by Coffin et al. (1). In their work, the death rate in control mice was 10.6%, in those exposed to 0.14 mg/m³ O₃ 20.0%, to 0.2 mg/m³ O₃ 35.0%, and to 0.98 mg/m³ O₃ 80.0%. Thus the reproducibility of this animal model system between two different laboratories and during two different time periods is apparent.

In an urban environment the concentrations of air pollutants vary considerably with their rate of emission and meteorological conditions. This frequently results in presence of low baseline concentrations of a pollutant with superimposed short-duration peaks of higher concentration of the same pollutant. For example, a large portion of nitrogen oxides present in an urban environment is generated by the vehicular traffic. Morning rush hour traffic results in high concentrations of nitric oxide and hydrocarbons which in presence of sunlight are converted to nitrogen dioxide. Subsequent sunlight irradiation of nitrogen dioxide results in increase in concentrations of ozone. Human populations therefore can be exposed over extended periods of time to low concentrations of NO₂ or to low concentrations of this pollutant with superimposed peaks of elevated concentrations of NO₂ and O₃ mixtures.

Figure 2 shows the effects of continuous 24 hours/day, 7 days/week exposure to 0.94 mg/m³ (0.5 ppm) or 2.82 mg/m³ (1.5 ppm) NO₂ on the susceptibility to K. pneumoniae infection (6). The increased mortality rates in mice exposed

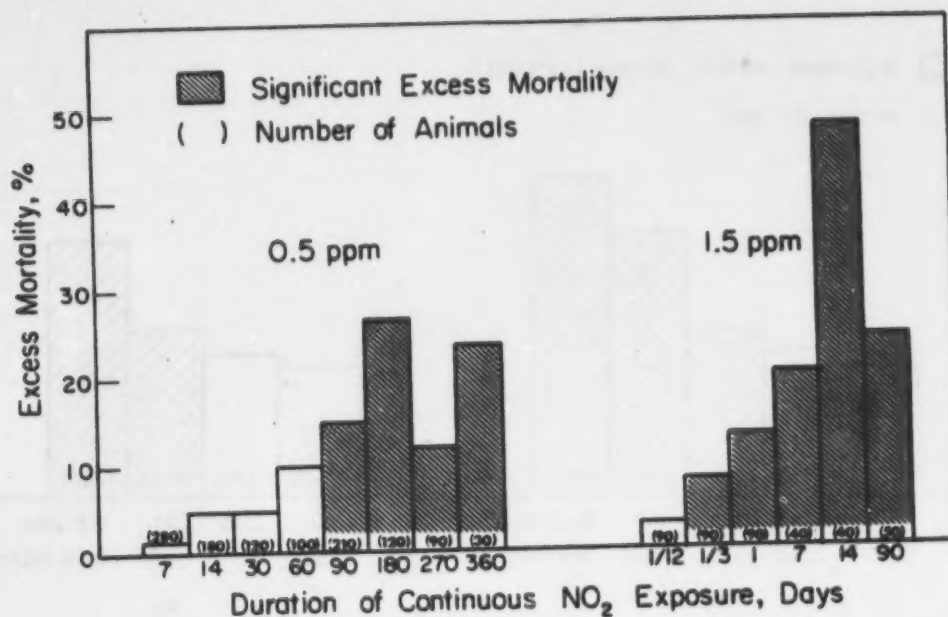


Figure 2. Excess mortalities in Swiss albino mice after chronic exposures to 0.5 ppm and 1.5 ppm NO_2 and challenged with *K. pneumoniae* (6).

to 0.94 mg/m^3 NO_2 for 3 months or longer, and to 2.82 mg/m^3 NO_2 for 8 hours or longer were significant ($p \leq 0.05$). Excess mortality was also present in mice exposed to nitrogen dioxide for the shorter time periods, but the differences were not statistically significant.

To determine the effects of inhalation of nitrogen dioxide and ozone mixtures, groups of mice were exposed for 3 hours to various concentrations of each pollutant, a mixture containing the same concentration of the two pollutants, or to filtered air. The four groups were then simultaneously challenged with *Streptococcus* aerosol. The differences between mortality among mice exposed to the pollutants and the corresponding control mice, challenged with the bacterial aerosol but exposed to filtered air, are shown in Table 1 (3). The numbers in parentheses show the mortalities expected to result from the exposure to the mixtures. The data indicate that the effects of the 3-hour exposure to the NO_2 and O_3 mixture were additive. In most instances the excess mortalities were equivalent or somewhat higher than the sum induced by exposure to each individual pollutant.

To further simulate the realistic condition of urban population exposure, groups of mice were exposed continuously 24 hours/day (7 days/week) to a background concentration of 0.19 mg/m^3 NO_2 with superimposed 3-hour daily peaks (5 days/week) of either 0.94 mg/m^3 NO_2 or a mixture consisting of 0.94 mg/m^3 NO_2 and 0.20 mg/m^3 O_3 . Inasmuch as under true environmental conditions

Table 1. Actual and Expected Changes in Mortality in Mice Exposed for 3 Hours to Nitrogen Dioxide and Ozone Mixtures and Challenged with Streptococcus Aerosol

Concentration			Mortality change, % ^a					
NO ₂		O ₃ ppm	0	0.05	0.1	0.5		
ppm	mg/m ³	mg/m ³	0	0.10	0.20	0.98		
			A	A (E)	A (E)	A (E)		
0	0		0	5.4	7.2	28.6*		
1.5	2.82		- 1.7	4.6 (3.7)	4.2 (5.5)	23.9* (26.9)		
2.0	3.76		14.3*	22.0* (19.7)	- (21.5)	56.2* (42.9)		
3.5	6.58		28.2*	- (33.6)	38.5* (35.4)	68.7* (56.8)		
5.0	9.40		35.7*	- (41.1)	- (42.9)	65.3* (64.3)		

^aPollutant mortality - control mortality. A - actual mortality change; E - expected mortality change.

Significant change in mortality compared to infected mice exposed to filtered air (*p ≤ 0.05).

exposure to the pollutants can be expected also to occur after the inhalation of the infectious bacteria, groups of mice were exposed to the pollutants before and for the 14-day period following the infectious challenge. Results shown in Figure 3 indicate that the combination of extended exposure to the pollutants and continuation of the exposure after the respiratory challenge with Streptococcus aerosol was most deleterious. Upon such an exposure sequence increased mortality and shortened survival time (see Figure 4) were seen in almost all exposure regimens. Moreover, these changes in resistance appeared sooner than when mice were exposed to clean air after the challenge. Another important factor appeared to be the stress of repeated daily exposure to the NO₂ and O₃ mixture. Irrespective whether or not the mice were reexposed to the pollutants or kept in clean air after the infectious challenge, increased mortality was seen in this group after 2 months or longer exposures.

The reduced mortality rates during the first 3 months of continuous exposure to 0.1 ppm NO₂ with the superimposed daily 3-hour peaks of either nitrogen dioxide and ozone mixture or 0.5 ppm NO₂ could possibly represent initial adaptation or a protective effect of the continuous exposure to nitrogen dioxide. This response occurred only after withdrawal from the polluted atmosphere and appeared to be negated by continuation of the exposure to the pollutants either for an additional 3 months or for the 14-day period after the infectious challenge.

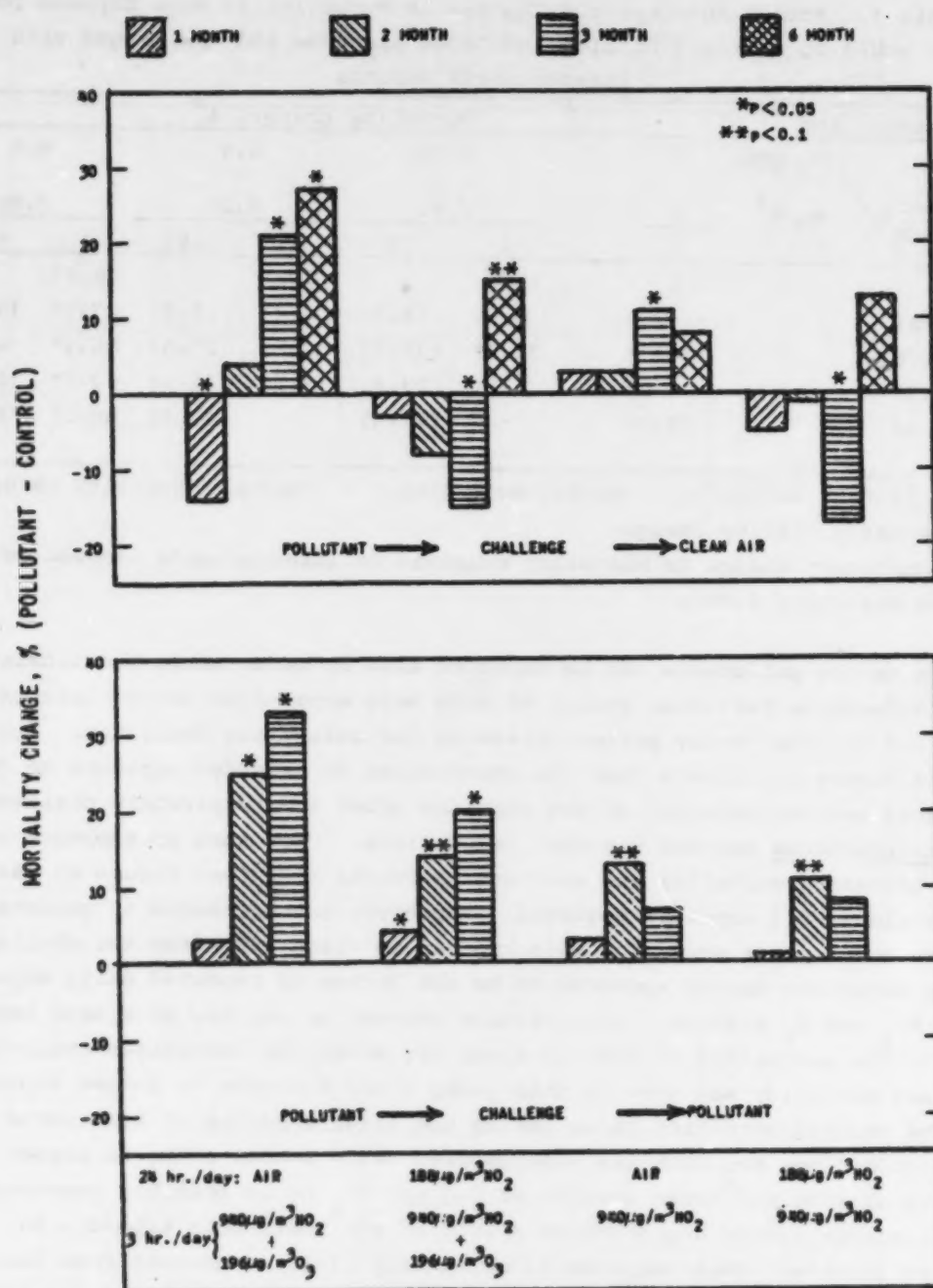


Figure 3. Changes in mortality from streptococcal pneumonia in mice subjected to various NO_2 and O_3 exposure regimens (5).

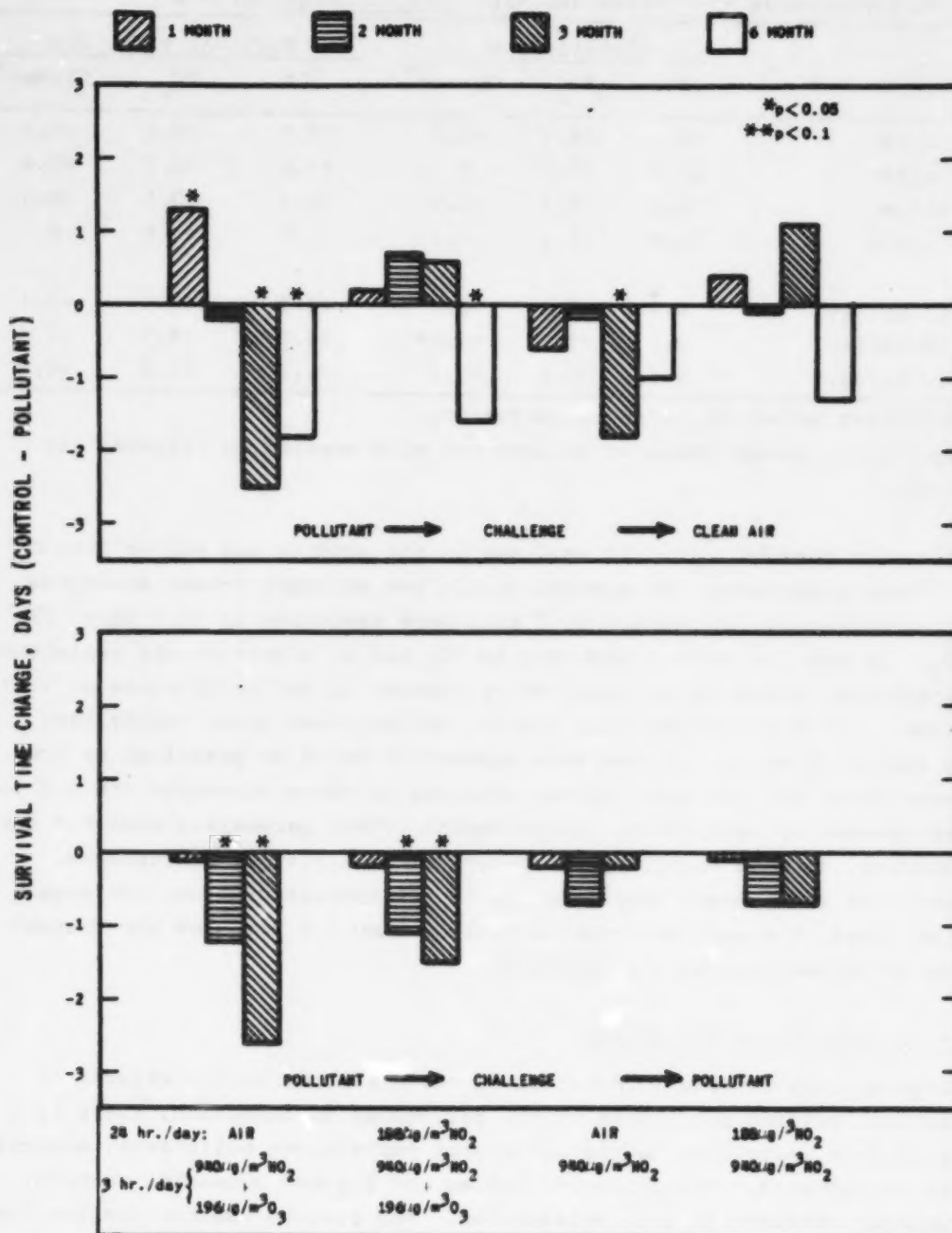


Figure 4. Changes in mean survival time in mice exposed to various NO_2 and O_3 regimens and challenged with Streptococcus aerosol.

Table 2. Mortality and Survival Time of Mice Exposed to 5.0 ppm (13.1 mg/m³) Sulfur Dioxide and Challenged with Streptococcus Aerosol

SO ₂ exposure	Mortality, %			Survival time, day		
	Air	SO ₂	Change ^a	Air	SO ₂	Change ^a
1 x 3 hr/day	24.1	26.1	+2.0	12.1	12.0	-0.1
5 x 3 hr/day	27.1	27.1	0	11.6	12.0	+0.4
10 x 3 hr/day	39.6	38.3	-1.3	10.4	10.5	+0.1
15 x 3 hr/day	31.9	30.4	-1.5	11.5	11.5	0
1 mo x 24 hr/day	9.9	7.8	-2.1	13.4	13.5	+0.1
2 mo x 24 hr/day	5.0	12.5	+7.5*	13.7	13.1	-0.6
3 mo x 24 hr/day	8.3	10.4	+2.1	13.4	13.3	-0.1

^aPollutant mortality - control mortality.

Significant change compared to infected mice exposed to filtered air (*p ≤ 0.05).

The third gaseous pollutant included in the studies was sulfur dioxide (SO₂). These experiments encompassed single and multiple 3-hour exposures as well as continuous 24 hours/day, 7 days/week exposures to 13.1 mg/m³ (5 ppm) SO₂. As seen in Table 2 exposure to SO₂ had no effect on the resistance to streptococcal pneumonia as measured by changes in mortality rates or survival time. The one statistically significant increase in mortality seen after 2 months of continuous exposure appears to be of no practical or true importance since all the other values obtained in these exposures did not indicate any changes in resistance. Other health effect parameters measured during these studies, such as viability and phagocytic activity of macrophages, development of lung edema, body weights, rectal temperature, and SEM examination of lungs, trachea, and nasal cavities, also did not show any changes which could be ascribed to the exposure.

Effects of Particulate Pollutants

Although considerable information is now available on the effects of photochemical oxidant pollutants on the resistance to infection, there is a paucity of data pertaining to the effects of particulate pollutants, especially sulfates or nitrates. The following tables and figures summarize results more recently obtained in our laboratories. The results further confirm the utility of this experimental animal model system for studies of air pollutants as well as environmental contaminants of importance to occupational health.

Table 3. Mortality and Survival Rate of Mice Exposed for 3 Hours to Sulfates and Challenged with Streptococcus Aerosol (4)

SO ₄ mg/m ³	ZnSO ₄			Zn(NH ₄) ₂ (SO ₄) ₂			(NH ₄) ₂ SO ₄		
	Mortality		MST	Mortality		MST	Mortality		MST
	D/T	%	day	D/T	%	day	D/T	%	day
0	373/1689	22.1	12.1	165/756	21.8	12.3	233/588	39.6	10.1
<1.1	125/599	20.9	12.2	29/120	24.2	11.9	22/48	45.8	10.1
1.2-2.0	369/813	45.4*	9.1*	112/445	25.2	12.0	76/191	39.8	10.7
2.1-3.0	186/278	66.9*	6.2*	67/192	34.9*	10.9*	52/96	54.2*	9.2
3.1-4.0	-	-	-	-	-	-	47/144	32.6	11.2
>4.1	-	-	-	45/48	93.8*	4.0*	46/110	41.8	10.3

MST - mean survival time.

Significant change from infected mice exposed to filtered air (*p ≤ 0.05).

Changes in susceptibility to streptococcal pneumonia caused by a single 3-hour exposure to zinc sulfate, zinc ammonium sulfate, and ammonium sulfate are shown in Table 3. Inhalation of 1.2 mg/m³ or more zinc sulfate or 2.1 mg/m³ or more zinc ammonium sulfate followed by a respiratory challenge with airborne Streptococcus resulted in significant increases in mortality and in reduced survival time in mice. Exposure to ammonium sulfate aerosol in concentrations up to 5.3 mg/m³ had no effect on susceptibility to the infection (4).

The changes in resistance to infection appeared to be related to the zinc ion present in the sulfate complex. The lesser effect of zinc ammonium sulfate than zinc sulfate in enhancing the severity of the infection and the absence of any effect of ammonium sulfate could be due to the presence of the ammonium ion which could possibly neutralize the effectiveness of the metallic cation.

The data shown in Figures 5 and 6 and Table 4 appear to confirm these assumptions. Figure 5 shows the least square lines, and the corresponding 95% confidence limit resulting from linear regression analysis of excess mortalities produced by a 3-hour inhalation of zinc sulfate and zinc nitrate. The similarity of the dose relation of these two compounds and their effectiveness in enhancing mortality caused by the infection are clearly seen. The correlation coefficient was highly significant (p ≤ 0.001) for mortality as well as the mean survival time (see Figure 6).

The possible neutralizing effect of the ammonium ion observed in the zinc ammonium sulfate was also seen in preliminary experiments with aluminum sulfate and aluminum ammonium sulfate. As shown in Table 4, exposure to 2.5 mg/m³ of aluminum sulfate resulted in a significant increase in mortality and decrease

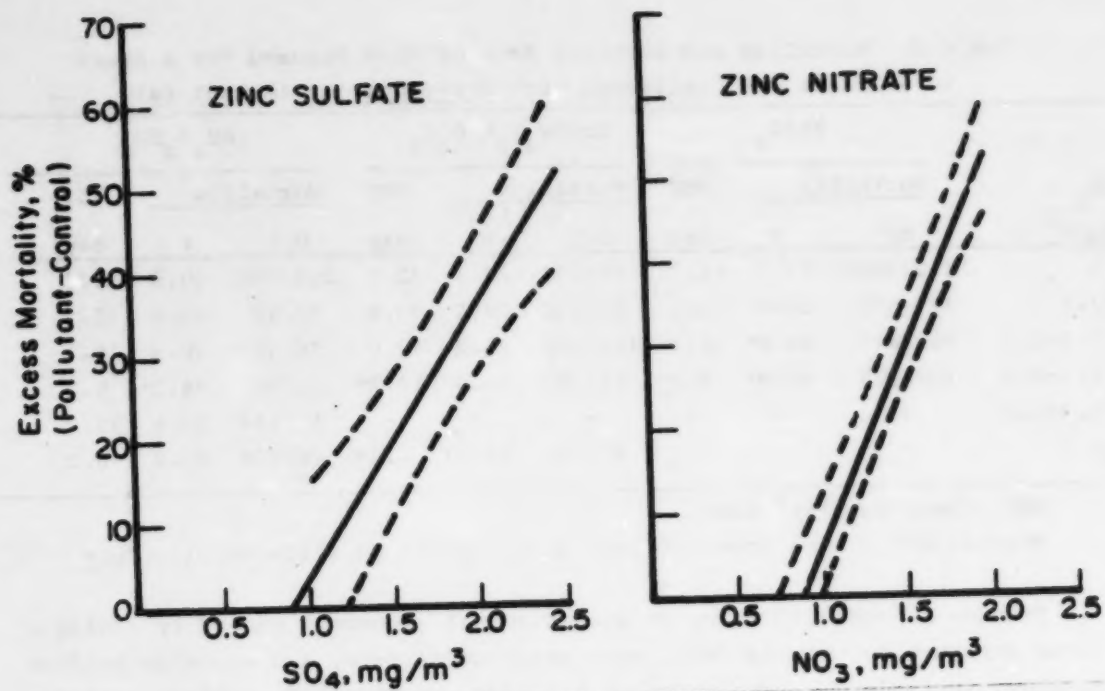


Figure 5. Excess mortality in mice exposed for 3 hours to zinc sulfate and zinc nitrate and challenged with Streptococcus aerosol.

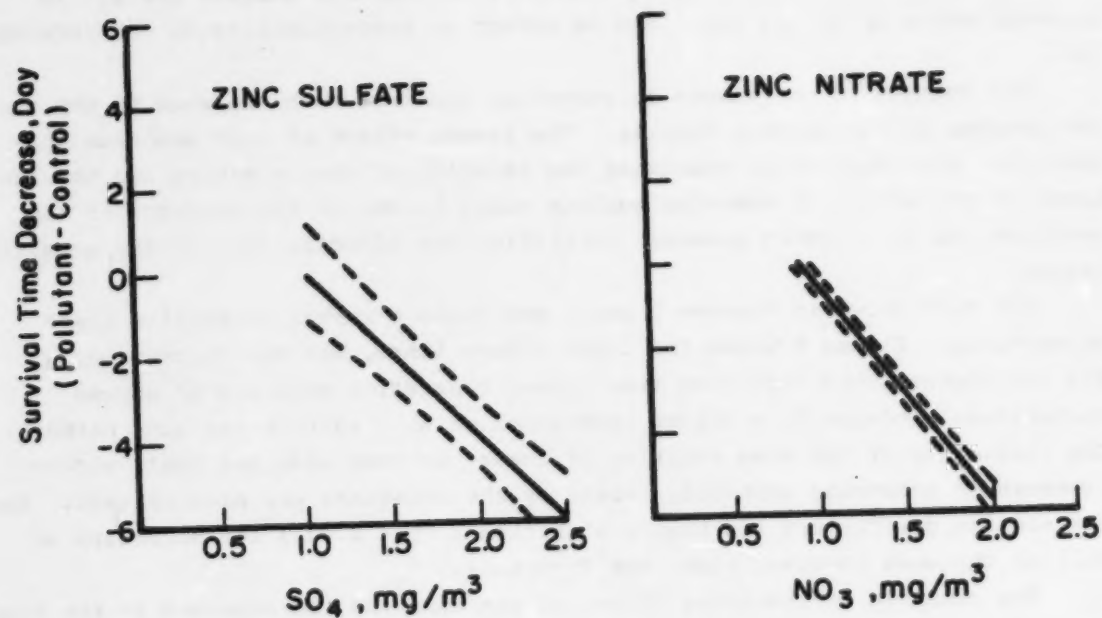


Figure 6. Changes in survival time of mice exposed for 3 hours to zinc sulfate and zinc nitrate and challenged with Streptococcus aerosol.

Table 4. Mortality and Survival Rate of Mice Exposed for 3 Hours to Sulfates and Challenged with Streptococcus Aerosol

SO ₄ ³ mg/m ³	Al ₂ SO ₄				AlNH ₄ (SO ₄) ₂			
	Mortality			MST day	Mortality			MST day
	D/T	%	Change		D/T	%	Change	
0	26/192	13.5	-	12.9	19/287	6.6	-	13.4
0.8	2/47	4.3	-9.2	13.8	-	-	-	-
1.5-1.9	-	-	-	-	11/144	7.6	+1.0	13.5
2.1	18/96	18.8	+5.3	12.6	11/96	11.5	+4.9	13.2
2.5	27/48	56.3	+42.8*	9.2*	6/48	12.5	+5.9	13.0

MST - mean survival time.

Significant change from infected mice exposed to filtered air (*p ≤ 0.05).

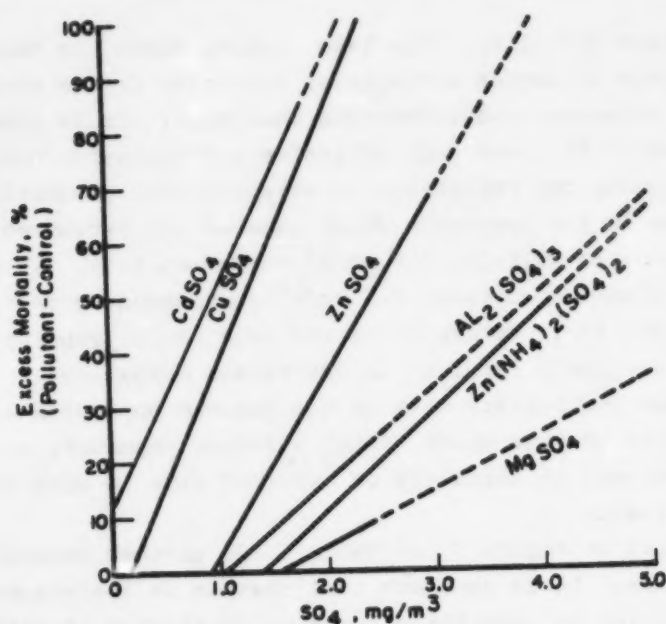


Figure 7. Excess mortality in mice exposed for 3 hours to sulfates and challenged with Streptococcus aerosol.

in mean survival time. On the other hand, a similar 3-hour exposure to the same concentration of aluminum ammonium sulfate resulted in somewhat increased mortality which, however, was not statistically significant.

Figure 7 shows the relationship between the concentration of several

Table 5. Maximum Tested Concentrations of Sulfates and Nitrates Which Did Not Alter the Resistance to Streptococcal Infection

Compound	mg/m ³
Aluminum ammonium sulfate - $\text{Al}(\text{NH}_4)(\text{SO}_4)_2$	2.4
Ferric ammonium sulfate - $\text{Fe}(\text{NH}_4)_2\text{SO}_4$	2.5
Ferric sulfate - $\text{Fe}_2(\text{SO}_4)_3$	2.9
Sodium sulfate - Na_2SO_4	4.0
Ammonium sulfate - $(\text{NH}_4)_2\text{SO}_4$	5.3
Ammonium bisulfate - NH_4HSO_4	6.7
Lead nitrate - $\text{Pb}(\text{NO}_3)_2$	2.0
Calcium nitrate - $\text{Ca}(\text{NO}_3)_2$	2.8
Sodium nitrate - NaNO_3	3.1
Potassium nitrate - KNO_3	4.3
Ammonium nitrate - NH_4NO_3	4.5

sulfates and excess mortality. The least square lines are based on linear regression analyses of excess mortalities resulting from a single 3-hour exposure to these compounds and infectious challenge. It is apparent from these data that cadmium sulfate was most effective and magnesium sulfate least effective in reducing the resistance to streptococcal pneumonia. The estimated concentrations of the compounds which induced 20% excess mortality (ED_{20}) were 0.2 mg/m³ cadmium sulfate, 0.6 mg/m³ copper sulfate, 1.5 mg/m³ zinc sulfate, 2.2 mg/m³ aluminum sulfate, 2.5 mg/m³ zinc ammonium sulfate, and 3.6 mg/m³ magnesium sulfate. Estimated on the same basis, exposure to 1.3 mg/m³ of zinc nitrate similarly resulted in 20% excess mortality.

Table 5 shows preliminary data on the maximum concentrations of sulfates and nitrates tested to date which, after a 3-hour exposure, did not result in significant changes in mortality or survival time in mice challenged with Streptococcus aerosol.

The data shown in Figure 7 and Table 5 add another dimension to this animal model system. It is apparent that changes in resistance to respiratory infection can be used to rank the effects of inhalation of pollutants having a related chemical structure. In all experiments the exposure conditions, including the particle size of the aerosol, were kept identical. Thus, the differences in response can only be ascribed to the toxicity of the compounds.

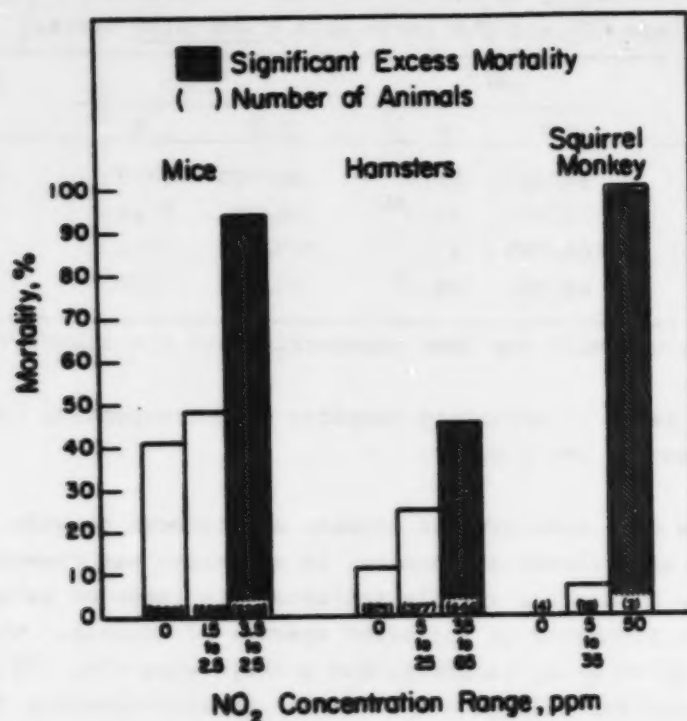


Figure 8. Effects of 2-hour acute exposure to NO₂ and challenge with *K. pneumoniae* on mortality of mice, hamsters, and squirrel monkeys (6).

EXPERIMENTAL VARIABLES

Several experimental variables can affect the responses in this animal model system. They include the animal host, infectious agent, and exposure sequences.

Animals

The selection of the animal host in this model system is of major importance. Figure 8 shows the response of mice, hamsters, and squirrel monkeys exposed to nitrogen dioxide and challenged with *K. pneumoniae* aerosol (6). The figure shows the percent mortality in the three species of animals and the numbers of animals used to obtain these means. Within each animal species, the mortality data were subdivided into three groups. The first is mortality of the control group of animals challenged with the infectious agent but exposed to filtered air. The second group represents mean mortality of animals challenged with the infectious agent and exposed to concentrations of nitrogen dioxide which did not significantly enhance the mortality. The

Table 6. Mortality in Different Mouse Strains after Exposure to 5 ppm NO₂ and Challenge with Klebsiella Aerosol

Strain	Air		NO ₂		Change
	D/T	%	D/T	%	%
BDF (black)	31/120	25.8 ^a	40/120	33.3	7.5 ^a
C57BL (black)	24/70	34.3 ^{ab}	36/70	51.4	17.1 ^{b*}
Swiss (albino)	164/390	42.1 ^b	262/390	67.2	25.1 ^{c*}
BALB (albino)	48/100	48.0 ^b	72/100	72.0	24.0 ^{c*}

Means in a column with the same superscript are not significantly different ($p \leq 0.05$).

Significant change in mortality compared to corresponding infected mice exposed to filtered air (* $p \leq 0.05$).

third group is the mean mortality of animals at nitrogen dioxide concentrations at which significant enhancement in mortality was observed.

The mortality of control animals indicates the range of natural resistance to bacterial pneumonia in different species of animals. The respective death rates in control mice, hamsters, and monkeys were 41%, 11%, and 0%. The respiratory dose for monkeys and hamsters was approximately 10⁵ organisms, a dose of the infectious agent which repeatedly killed the Swiss albino mice. Thus, the higher concentrations of nitrogen dioxide necessary to induce a significant increase in mortality in monkeys and hamsters can, at least in part, be ascribed to their innate resistance to this infectious agent.

Differences in natural resistance to bacterial pneumonia were also seen in studies of the effects of nitrogen dioxide in four strains of mice and are shown in Table 6. The BDF mice showed significantly higher natural resistance to the infection than the other strains. However, excess mortalities ranging from 8 to 25% were observed in all four strains of mice after 3-hour exposure to 5 ppm of NO₂ and the infectious challenge.

Infectious Agent

Table 7 shows the effects of a single 2- or 3-hour exposure to nitrogen dioxide on the resistance of Swiss albino mice to bacterial pneumonia induced by inhalation of K. pneumoniae or S. pyogenes aerosols. A direct comparison between the two groups of results cannot be made because of the time interval separating the two studies. The work with Klebsiella was reported in 1966 (2), the one with Streptococcus in 1977 (3). Nevertheless, it is apparent that the mice were somewhat more susceptible to the Klebsiella than to the Streptococcus infection.

Table 7. Effect of a Single Exposure to Nitrogen Dioxide on Mortality of Mice Challenged with *Klebsiella* or *Streptococcus* Aerosol

NO ₂ Concn.		Mortality change, % ^a	
ppm	mg/m ³	<i>Klebsiella</i> ^b	<i>Streptococcus</i> ^c
1.5	2.82	+10.0**	-1.7
2.0	3.76	-	+14.3*
2.5	4.70	+5.7**	-
3.5	6.58	+54.0*	+28.2*
5.0	9.40	+48.8*	+35.7*

^aPollutant mortality - control mortality.

^bTwo-hour exposure to NO₂.

^cThree-hour exposure to NO₂.

Significant change in mortality compared to infected mice exposed to filtered air (*p < 0.05; **p < 0.1).

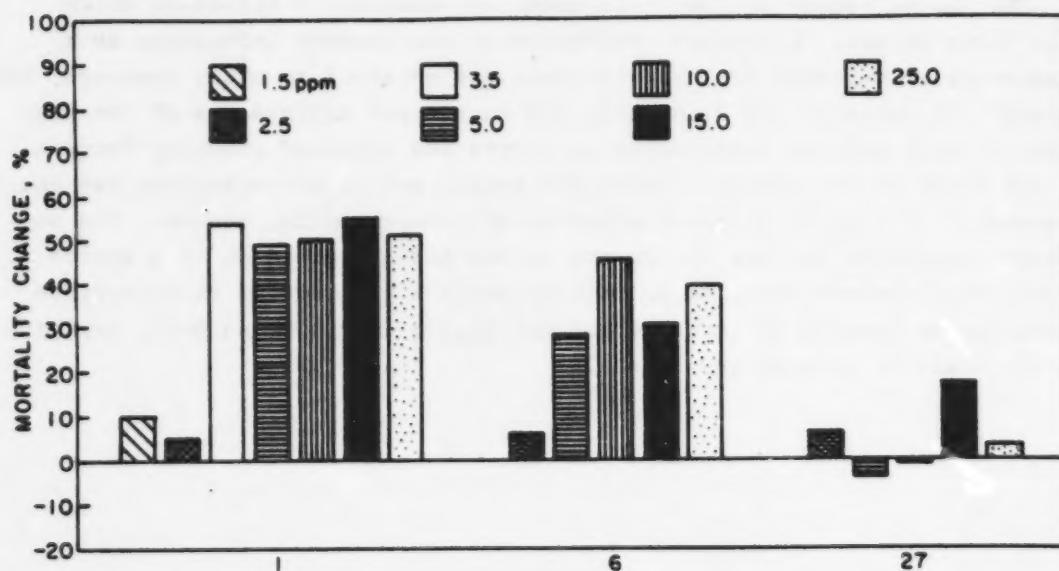


Figure 9. Time interval between 2-hour exposure to NO₂ and infectious challenge, hours.

Exposure Sequence

The sequence and interval between the exposure to the pollutants and challenge with the infectious agent are yet another factor of importance. In all of the studies discussed previously, the experimental plan required exposure to the pollutants followed within less than one hour by the infectious challenge. Figure 9 shows that, at least for the short-term exposures to nitrogen dioxide, the extension of the interval between exposure and challenge allows a recovery from the effects of the pollutants.

CONCLUSIONS

In discussing the importance of this animal model system in studies of health effects of air pollutants two aspects must be considered. First is the value of the model as a highly sensitive indicator of in vivo effects. The changes in resistance to respiratory infection can be seen at concentrations of oxidant and other pollutants markedly lower than those detected by any other in vivo method. The model depicts the responses of the respiratory system and reflects the overall damage to the pulmonary defense mechanisms. Therefore, it indicates that basic defense mechanisms of the lungs have been impaired. A major part of this impairment appears to result from the damage to the alveolar macrophage system and the ensuing inability to remove the inhaled microorganisms from the lungs.

The second aspect is that this model may simulate a situation which could occur in man. Decreased resistance to respiratory infections as a consequence of exposure to air pollutants was observed in mice, hamsters, and monkeys. In spite of the functional and anatomical differences of the respiratory tract and the differences in innate and acquired immunity between man and these animal species, under the proper set of circumstances man could be expected to respond to these stresses in a very similar manner. The necessary components for the occurrence in man are the presence of a sufficiently high concentration of pollutants and the presence of an infectious microorganism capable of invading and colonizing in the human host, exploiting the state of reduced resistance.

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DISCUSSION

DR. DREW: What was the basis for the selection of 5 ppm for SO₂? I noted that in several of your experiments you were looking at 5 ppm ozone and SO₂, knowing that the toxicities of these compounds are different.

DR. EHRLICH: It was just an arbitrary selection which was in part guided by the SO₂ limits in the atmosphere as suggested by the EPA. It was not a scientific or technical selection, but simply a practical selection to determine the effects of SO₂ at this concentration.

DR. DREW: In that last slide it appeared that there is no dose response, that there is a very flat dose response essentially, once you get above 3.5 ppm of nitrogen dioxide. Regardless of what the concentration was, the percent in mortality did not increase any further.

DR. EHRLICH: The slide showed excess mortalities rather than actual death rates.

DR. DREW: I understand, but they were all the same after 3.5 ppm, at least in the first column of that last slide.

DR. EHRLICH: This is rather old data obtained with K. pneumoniae. We found that with K. pneumoniae we could not obtain a meaningful dose response, inasmuch as this agent is highly infective to mice, and mice appear to be highly susceptible. One of the reasons we changed to Streptococcus sp. was because of this. It's a much more reproducible infectious system providing a better dose-response mortality rate.

DR. DAWSON: One of the problems that's been raised in connection with the preparation extrapolating to a human case is the number of organisms that are delivered. Can you comment on that?

DR. EHRLICH: I never suggested to use this model for direct extrapolation to man. On the other hand, I feel strongly that it's a highly sensitive model, which presents a total biological response reflecting the various defense mechanisms involved. I think that it is the only model which, in a

way, puts all those things together. As far as the human infections are concerned, I think they are different, in terms of man's susceptibility to infection. Thus I don't think we could expect to see the same type of response in humans. Your criticism is valid only if one just wants to take the laboratory information and extrapolate it directly into man. I am presenting it as a highly sensitive biological model system most appropriate to studies of health effects of pollutants. The data on SO_2 and some of the sulfates and nitrates are important and reinforce the validity of this model system because they demonstrate that the model does not respond to every pollutant. It certainly responds only to compounds which affect the resistance to infection.

Experimental Infection of the Respiratory Tract with *Mycoplasma pneumoniae*

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Mycoplasma pneumoniae is one of the most common pathogens of the human respiratory tract. Infection with this agent is the leading cause of acute pneumonitis in high contact environments such as college campuses and military bases, as well as being a common cause of respiratory illness in children (6). Illnesses associated with M. pneumoniae infections range from mild upper respiratory infection, through tracheobronchitis, to acute pneumonia (2). These agents have been associated with exacerbations of chronic bronchitis in adults with chronic obstructive pulmonary disease and with severe illnesses in compromised hosts such as patients with B-cell immunodeficiency (8) or sickle cell anemia (19). Because of the prevalence and seriousness of M. pneumoniae associated respiratory illness, it is important to determine the effects of air pollution on the interaction between this organism and host respiratory tissues. Fortunately, the Golden Syrian hamster provides an established animal model for M. pneumoniae infection (5). The purpose of this paper is to review briefly the features which have made this animal model so ideal for studying M. pneumoniae pathogenesis, to describe a recently developed method for studying this model using aerosolized, radioactively labeled M. pneumoniae, and finally, to describe some observations on the deposition and clearance of aerosolized mycoplasmas within hamster lungs.

REVIEW OF ANIMAL MODEL MYCOPLASMA PNEUMONIAE INFECTION

Studies on the pathogenesis of M. pneumoniae infection in the Golden Syrian hamster have been conducted both in vitro and in vivo. One main thrust of the in vitro work has involved the study of isolated tracheal rings infected with M. pneumoniae (3, 4). Through these studies it has been established that M. pneumoniae is a filamentous organism, lacking a cell wall, but possessing a unique tip structure responsible for its attachment to its primary host target tissue, ciliated respiratory epithelium (4). This attachment causes a variety of adverse effects on the ciliated cell, including alterations in cellular metabolism (10, 11), changes in cellular ultrastructural morphology (4), and eventually, disorganization of ciliary activity and loss of cilia from the epithelial surface (15).

Additionally, examination of the in vitro interaction between M. pneumoniae and isolated alveolar macrophages (16, 17) has shown that M. pneumoniae, like several other mycoplasma species (14), has the capacity to resist phagocytosis unless opsonized with specific antibody or complement. This feature exists despite the absence of an identifiable capsule or cell wall, the key components essential for bacterial antiphagocytic capacity.

Studies of the effects of M. pneumoniae infection in the Golden Syrian hamster began with the work of Dajani et al. (5). Following intranasal inoculation of 0.2 ml of virulent organisms, the hamster develops a bronchopneumonia which is very similar to human disease. At the peak of infection, the histologic picture consists of scattered areas of peribronchial and to a lesser extent perivascular round cell infiltrate. The intraluminal exudate which develops is composed of mononuclear and polymorphonuclear phagocytes (1). Pulmonary disease is evident by the third to seventh post-infection day, peaks on the tenth to fourteenth day, and begins to resolve by the third week of infection. All animals ultimately recover from the primary infection with a return to normal of the lung histology and complete clearance of the organisms. The development of IgG antibodies, as measured by complement fixation and growth inhibition, follows a time course similar to that of human disease. When animals have recovered from a primary infection, they are left with a long-lasting relative immunity against reinfection. While the precise mediators of this immunity remain undefined, it is known that stimulation of local pulmonary immune mechanisms is more important than stimulation of systemic immunity (7).

This concept was substantiated following the recent article by Jemski et al. (13), outlining the role of aerosol particle size and site of deposition of M. pneumoniae on the development of respiratory disease and immunity in hamsters. In their report, representing the only published account of an animal model aerosol infection by M. pneumoniae, the authors demonstrated that the response of hamsters to M. pneumoniae infection was determined by the site of

deposition of organisms and by the quantity of organisms introduced directly into the lower respiratory tract. The variables which were studied included M. pneumoniae delivered as a large particle aerosol, with a mass median diameter of 8 μ m, a small particle aerosol with a mass median diameter of 2.3 μ m, or intranasal inoculation of 200 μ l, 20 μ l, or 2 μ l of organisms. Only the large volume intranasal inoculum and the small particle aerosol resulted in deposition of mycoplasmas into the lungs. The other three routes of infection colonized only the upper airways. The most significant disease, as measured by the percentage of animals with pneumonia and overall mean pneumonia score, was found in animals with the large volume intranasal inoculum. Animals receiving small volumes but containing the same number of mycoplasmas, administered only to the upper airway, developed little or no pneumonia. This observation suggested to these authors that the development of disease in the hamster may depend largely on the initial number of organisms delivered directly to the lower respiratory tract. Thus, it would seem that initial deposition and rate of organism clearance may be very important determinants of disease development.

Similarly, the initial location of organism deposition within the respiratory tract was critical in the generation of a protective immune response. In this segment of the study, all animals were allowed to recover 4-8 weeks following primary infection and then were challenged with a large volume inoculum of intranasal organisms. While any route of infection conferred at least some protection against disease development, maximal protective immunity was seen only in those animals given initial exposure which provided inoculation of the lower respiratory tract--that is, either by large volume intranasal inoculation or by small particle aerosol. Thus, again, initial deposition and pulmonary clearance would seem to be crucial events in determining the generation of protective immunity.

Aerosol Chamber Development

With these data in hand, we decided that the evaluation of air pollutant effects on disease and immune response to M. pneumoniae infection in the hamster must be studied such that the initial deposition and clearance could reproducibly be evaluated. To accomplish this, an aerosol exposure system was developed in which viable mycoplasmas, tagged with a radioactive marker, could safely and efficiently be delivered in an aerosol with a particle mass diameter that would assure deposition deep within the lower respiratory tract. One of the main criteria considered in designing the aerosol inhalation chamber was to be able to have a high concentration of radioactively labeled M. pneumoniae introduced into the lungs to make deposition and clearance studies possible. Thus, we designed a chamber of small volume with a low total air flow rate to maximize the organisms' air concentration during a 25-minute exposure period. Figure 1 shows

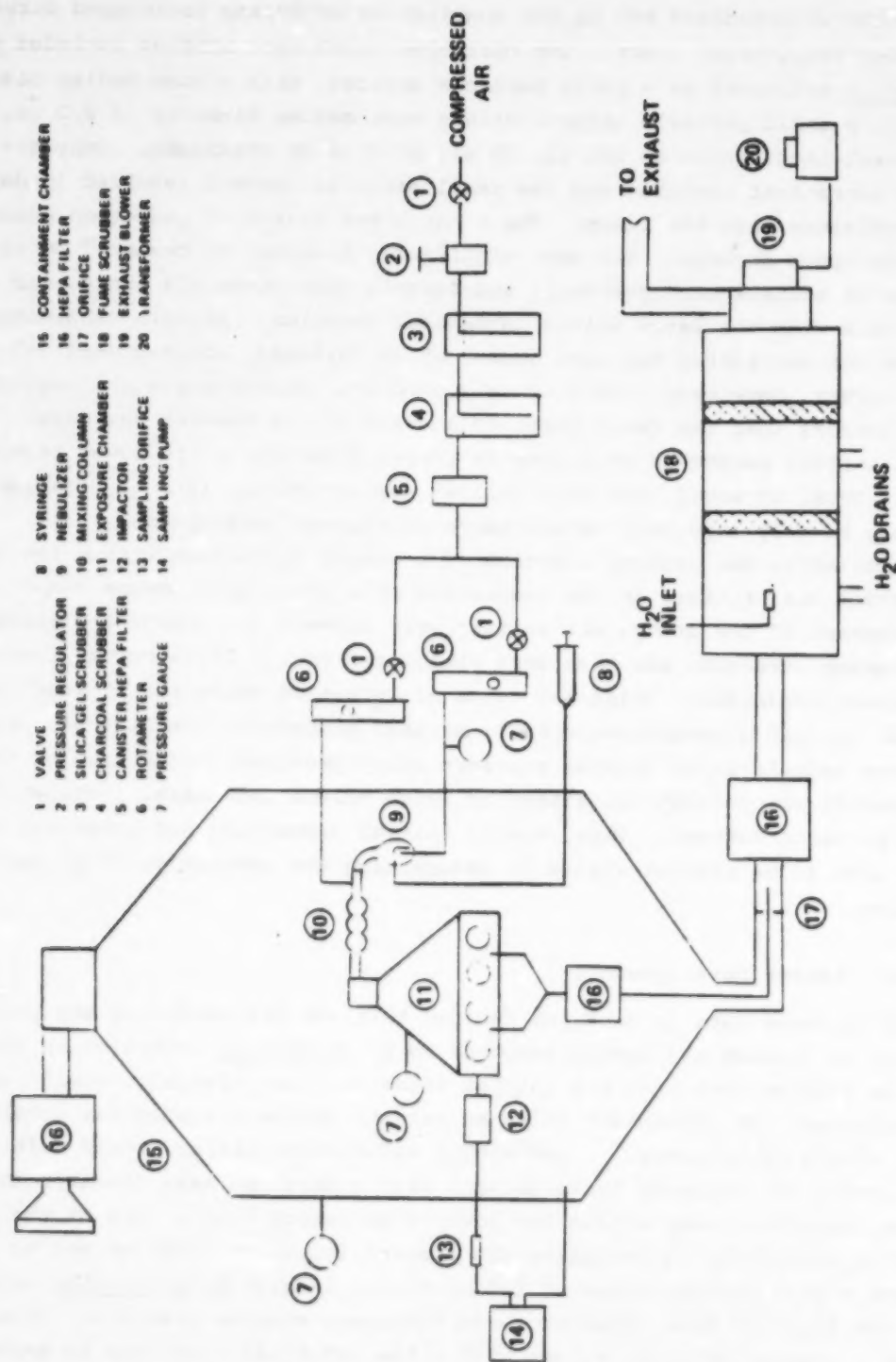


Figure 1. Schematic diagram of Mycoplasma pneumoniae aerosol inhalation chamber.

a schematic diagram of the inhalation chamber. Compressed air is filtered and passed into rotometers which control air flow rate into the nebulizer and the drying column. Air enters this column through two tangential inlets causing a turbulence which causes evaporation of water from the nebulized aerosol reducing the particle size. From the drying column, the combined air streams enter the center of the chamber and then flow into the two side exposure sections where inhalation occurs.

Since M. pneumoniae are human pathogens and were radioactive in many experiments, stringent safety precautions were taken to filter all exhaust air and provide adequate containment of the aerosolized organisms. This was accomplished by housing the nebulizer, mixing column, and exposure chamber inside a stainless steel Rochester chamber, and venting all exhaust air through several HEPA particle filters. Sampling of the aerosol particle size was performed with a seven-stage low flow rate cascade Lovelace impactor.

To prepare radioactively labeled M. pneumoniae for aerosolization, organisms were grown on the inner surface of glass bottles, labeled with tritiated oleic acid, washed extensively to remove unincorporated radiolabel, and scraped into M199 containing 1% horse serum. During a single run, 4 ml of suspended organisms containing approximately 10^{10} colony-forming units (CFU) were aerosolized over a 25-minute period at 21°C and 27% relative humidity. Following this, 4 ml of saline were aerosolized for an additional 25 minutes to complete the total exposure. To attain the desired particle size, the nebulizer flow rate and dilution air flow rate were 5.5 and 11.5, respectively.

Table 1 shows the high degree of particle size reproducibility accomplished with this chamber. Here, results of experiments occurring over a one-year period are presented to demonstrate the consistency of the count median aerodynamic diameter. The latter value, reflecting the particle size reaching the hamster lung, is within the size range which would assure deposition within the distal airways of the lower respiratory tract.

The correlation between the radioactive concentration in the nebulizer solution and the radioactive counts measured within the central aerosol chamber and found in the hamsters' lungs at the end of aerosol exposure is shown in Table 2. As shown by the regression analyses at the bottom of the table, there was greater than 90% correlation between the concentration of mycoplasmas within the chamber air and that found in hamster lung.

Of potentially more importance, however, was the similarity of deposition for all animals during each aerosol exposure. If any ports were found to provide variable exposure or exposure consistently above or below the mean, the value of this exposure chamber for comparative studies would be questionable. Figure 2 demonstrates graphically the excellent correlation of exposure in all eight animals studied in each of four successive experiments. These data are tabulated in Table 3 where it can be seen that the coefficient of variation of

Table 1. Size Distribution Data for M. pneumoniae Aerosol
in 8-Port Chamber

Exposure date	Count median aerodynamic diameter (CMAD)	Geometric standard deviation (gg)	Mass median aerodynamic diameter (MMAD)
08-19-77	1.8	1.5	2.9
10-11-77	1.6	1.4	2.2
10-20-77	1.7	1.4	2.5
10-25-77	1.7	1.4	2.5
12-01-78	1.7	1.5	2.7
01-17-78	1.4	1.4	2.0
01-19-78	1.4	1.5	2.1
01-21-78	1.3	1.4	1.9
08-04-78	1.5	1.4	2.2
08-07-78	1.5	1.4	2.1
08-14-78	1.6	1.4	2.2
08-16-78	1.6	1.4	2.3
Average $\pm$ S.D.	1.57 $\pm$ 0.15	1.42 $\pm$ 0.05	2.3 $\pm$ 0.3

the average cpm/mg of dry lung weight for the eight animals in any one experiment varied from 0.14 to 0.19.

One important question in an aerosol system such as this is the extent to which aerosolized organisms remain viable. To determine how many organisms were killed by the aerosolization process, the percentage of mycoplasmas found to be viable upon deposition in the hamster lung immediately at the end of aerosol exposure was multiplied by the specific activity (CFU/cpm) of the initial aerosol suspension to determine the theoretical number of viable organisms delivered to the respiratory tract. The CFU of organisms recovered divided by this theoretical number revealed the approximate actual survival rate which averaged 55%.

DEPOSITION AND CLEARANCE OF MYCOPLASMA PNEUMONIAE IN THE HAMSTER LUNG

Following the achievement of technical reproducibility in the mycoplasma aerosol chamber, clearance studies were undertaken in nonimmune hamsters. In these studies, pulmonary clearance, as measured by the removal of radioactively tagged mycoplasmas from the lung, was compared with pulmonary mycoplasma capacity, as measured by the rate of decline of mycoplasma viability within the lung. One aim in these studies was to compare the clearance and

Table 2. Comparison of Mycoplasma pneumoniae Concentrations in Nebulizer Solution, Air Sample, and Hamster Lungs

Exposure date	Nebulizer solution concentration, cpm/10 μ l	Air sample concentration, cpm/ml air	Average hamster lung content at To, cpm/lung
8-19-77	2.5×10^5	15.6	6,241
8-22-77	0.85×10^5	4.4	2,358
8-31-77	2.4×10^5	13.6	6,097
9-08-77	2.8×10^5	32.1	13,135
10-11-77	1.2×10^5	8.9	4,453
10-13-77	1.2×10^5	7.9	4,210
10-20-77	1.2×10^5	-	2,403
10-25-77	2.2×10^5	13.4	6,138
12-01-77	0.94×10^5	-	6,807
01-17-78	1.2×10^5	-	5,424
01-19-78	1.0×10^5	12.6	5,661
01-21-78	0.78×10^5	6.9	4,815
08-04-78	3.6×10^5	41.5	15,937
08-07-78	4.5×10^5	39.6	20,283
08-14-78	3.0×10^5	26.7	15,581
08-16-78	3.2×10^5	-	22,010

Regression Analysis		Y = A + BX	r = correlation coefficient
X vs. Y	A	B	r ²
Nebulizer solution vs. lung content	-995	0.0483	0.76
Nebulizer solution vs. air concentration	-3.08	1.00×10^{-4}	0.84
Air concentration vs. lung content	719	431	0.92

viability curves with similar data published on animal models infected with aerosolized bacteria (9, 18). Prior studies have examined in vivo clearance of radioactively labeled bacteria administered to animal lungs by aerosol exposures which provide particles of approximately 2 μ m mass median diameter. Under these circumstances, deposition occurs primarily at the alveolar level and

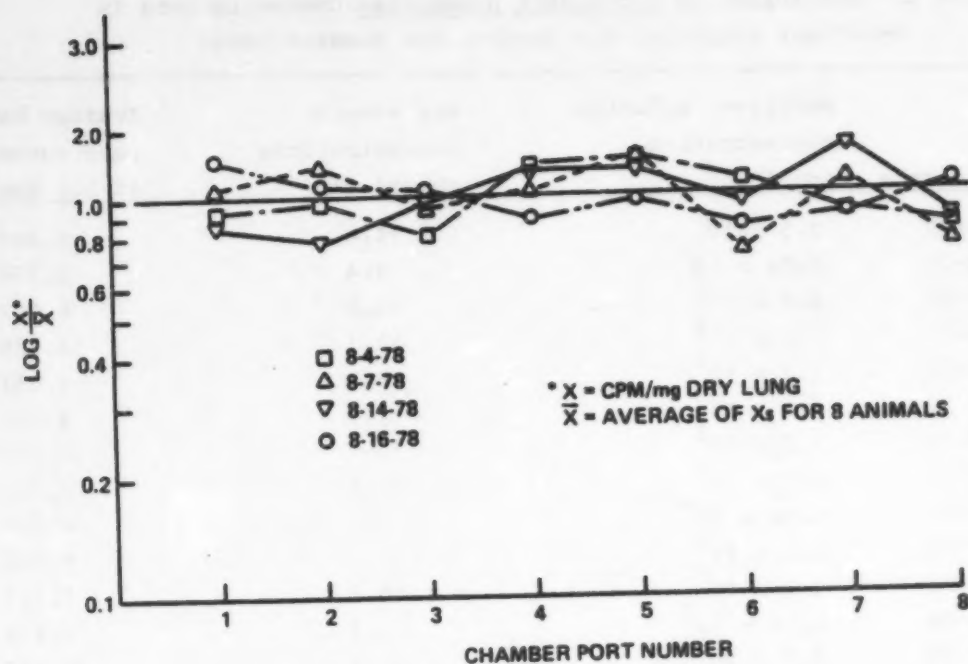


Figure 2. Chamber position vs. normalized deposition of radioactively labeled *Mycoplasma pneumoniae*.

Table 3. Specific Activity of Radioactively Labeled *Mycoplasma pneumoniae* Deposited in Hamster Lungs

Exposure date	Average cpm for 8 animals	Average cpm/mg dry lung for 8 animals	Standard deviation of individual cpm/mg dry lung	Coefficient of variation
8-04-78	15,937	122.5	19.8	0.16
8-07-78	20,283	166.9	32.0	0.19
8-14-78	15,581	119.0	22.6	0.19
8-16-78	22,010	162.4	22.2	0.14

provides the capacity to analyze simultaneously physical removal and viability of the intra-alveolar organisms.

With most bacteria, the rate of transport or physical clearance of radioisotope out of the lung occurs more slowly than the decline in the number of viable bacteria. This has led to the theory that physical removal of bacteria plays a relatively minor role in the early defense of the lung and that a more important pulmonary defense mechanism involves alveolar macrophage engulfment and intracellular killing of the inhaled bacteria (12).

Figure 3 shows the viability of M. pneumoniae deposited in the total lung tissue. There was little decline in the viability of M. pneumoniae during the first eight hours after aerosolization. Only after this time period was there a decline in viability such that by 24 hours the viable mycoplasmas in the lung were reduced by 90%. Thus, during the initial eight hours, when macrophage engulfment and intracellular killing play the chief role in intrapulmonary removal of inhaled bacteria, little change in M. pneumoniae viability was noted. However, the number of viable organisms began to increase significantly by 48 hours

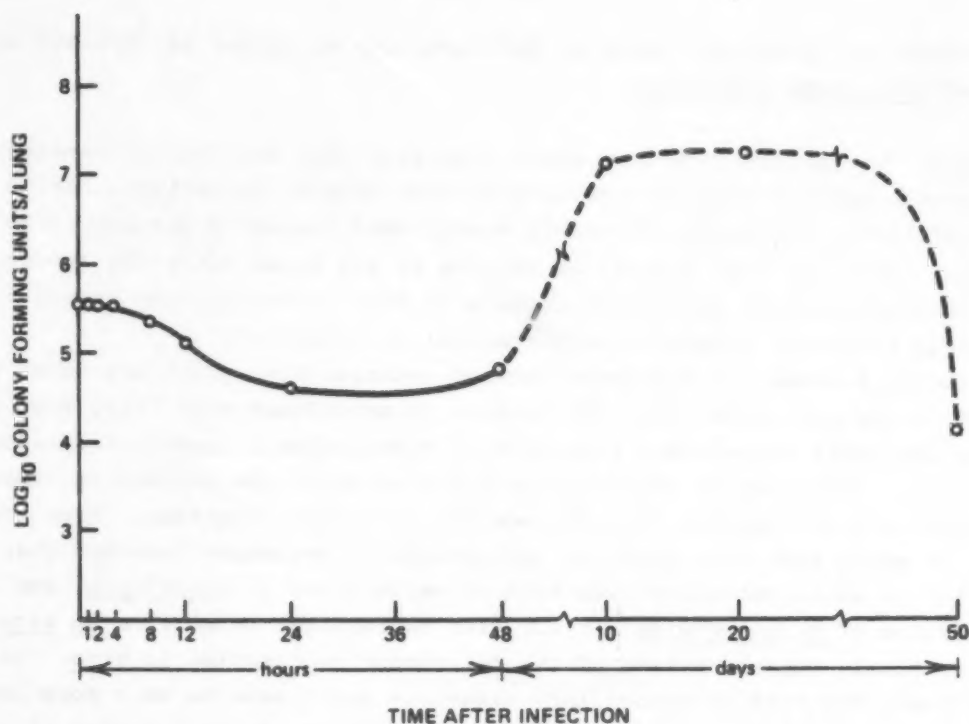


Figure 3. Clearance of viable Mycoplasma pneumoniae from hamster lungs following aerosol infection.

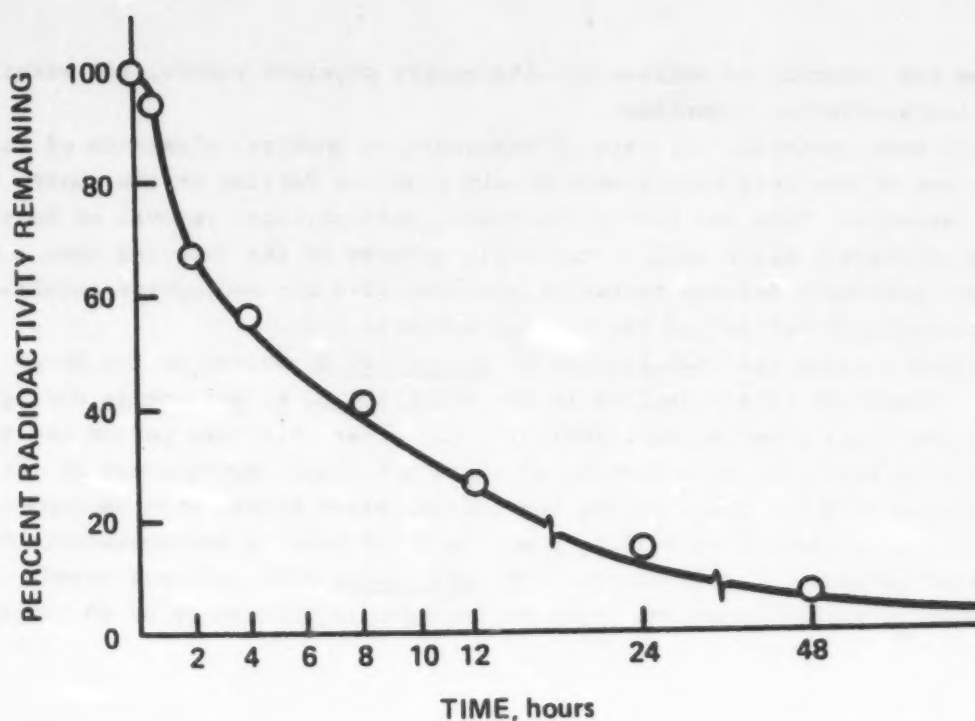


Figure 4. Clearance curve of radioactivity deposited as ^3H -oleic acid-labeled Mycoplasma pneumoniae.

following the initial infection which indicates that the rate of replication was faster than the rate of clearance by host defense mechanisms. At 10 days post-infection, the number of viable mycoplasmas reached a plateau of 10^7 organisms per lung, and then started to decline by six weeks after the infection. Histological sections taken from animals 20 days post-infection showed typical pathology including extensive peribronchial infiltration.

Figure 4 shows the clearance rate of radioisotope, which was found to be significantly more rapid than the decline in mycoplasma viability, most likely due to the rapid mucociliary clearance of nonattaching, inactivated aerosolized organisms. There was no point on the curve at which the percent of remaining radioactivity was greater than the percent of viable organisms. From these data, then, it would seem that alveolar macrophages in nonimmune hamsters play a limited role in the early pulmonary clearance of aerosolized M. pneumoniae, and the interaction of M. pneumoniae and alveolar macrophages noted from in vitro studies is most likely representative of the relationship occurring in vivo. On the other hand, the role of mucociliary clearance would seem to be a more important nonspecific host defense mechanism in minimizing the initial number of viable organisms capable of establishing residence within the host lung tissues.

With the establishment of this technique for the reproducible aerosolization of viable, radiolabeled M. pneumoniae we are encouraged that many features of this important respiratory infection can be examined in the hamster animal model and the adverse effects of air pollution exposure determined. In addition to the ready measurement of deposition and clearance with this system, we have demonstrated that aerosol infected hamsters eventually develop histologic changes and serologic responses comparable to animals infected by 0.2 ml intranasal inoculation. Thus, air pollutant effects on the development of this form of chronic pneumonia, as well as on the generation of protective immune response, will also be among our future research objectives.

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DISCUSSION

DR. JAKAB: I'd like to propose another reason why the curves differed. First of all, you used a different tracer than we did, and second of all, your animals are restrained, they incubate in the aerosol chamber. This morning Joe Brain presented some very nice studies showing that if you have a shallow breathing animal--a rabbit is a shallow breathing animal--whatever was deposited was primarily in the bronchi, whereas with deep breathing it would go in the lung parenchyma. I was wondering whether restraining affected the breathing pattern that Joe showed, and therefore your animals inhaled mycoplasma and that position was preferentially in the bronchi, and therefore (inaudible) did not reach the alveoli.

DR. POWELL: It's certainly a possibility. We have autoradiographs, which we're waiting to develop, to determine the site of deposition in the alveoli.

DR. HU: We also did some experiments not shown here where we actually take out the individual lobes and determine radioactivity. We see that there is a constant, even distribution of radioactivity in all parts of the lung.

DR. DAWSON: I'm still worried about this question of extrapolation to man. You started off by saying that hamsters were extremely good experimental models, and I wonder why that is. What is so good about hamsters? They're extremely resistant compared to squirrel monkeys. Are these levels of exposure of organisms that you were using like those man might get in his environment?

DR. POWELL: Let me start by answering the first question. I think that a hamster is fortuitous in the sense that it does take a human pathogen. Relatively few mycoplasmas are required to be deposited in the lung to develop a pneumonia along a similar time course thought to be due to the incubation in humans, that is, two weeks. We have a limited knowledge of histology of mycoplasma disease in humans because it's rarely a fatal disease. When compared to the pathology in hamsters, it's basically the same kind of histologic picture, that is, of peribronchial round cell pneumonia. Serologically, the time course is almost identical to that in the human. It mimics human disease with the exception that the hamster does not become overtly ill. In that sense, it provides a very good model to manipulate.

Is it artificial to be aerosolizing? Is it more appropriate to be putting the stuff right in the nose? Do all infections occur with a primary upper respiratory infection that leads then only to the lower respiratory tract? I don't think that we really know the answer to that, to be quite honest. In fact, the paper that I cited regarding infection and immunity would say that at least in the hamster that is not the case. If you deposit large numbers of organisms in a very small dose, they stay limited to the upper airway, they do not progress to involve the lower respiratory tract. There are some fascinating fortuitous studies in humans in dental clinics where people grind the teeth of individuals with mycoplasma infection. This generated an outbreak of M. pneumoniae disease in people working in the clinic, suggesting that aerosol infection is probably the most efficient way to infect the human host as well. In that sense this may not be all that far from the way much of human disease originates.

DR. DAWSON: How about numbers of organisms?

DR. POWELL: I don't know what kind of numbers of organisms it takes to cause a primary infection in the human. We do know that infections occur repeatedly in humans, and of those repeated infections few probably cause disease. Whether many of those occur at a subliminal dose or whether they require a certain number of infections to generate immune responses leading to disease just is not known. Clearly, there are a lot of infections that are subclinical or cause very mild upper respiratory illness.

DR. GARDNER: I might mention in the bacterial model system that it takes only between 400 and 4,000 microorganisms in the lung at zero time to show the enhancement of mortality.

PARTICIPANT: In mice?

DR. GARDNER: In mice.

DR. COFFIN: In any species you have to have enough organisms to cause the disease. If you can then do some other manipulations to make them more susceptible, a greater number of animals should have that disease regardless of the number of organisms required. For instance, in Streptococcus of human variety, it is impossible to infect a mouse by inhalation. You can show that a mouse becomes more susceptible to the human Streptococcus, but it takes more of the secondary problem, the ozone or whatever you're using, because the mouse is not as susceptible to human Streptococci. What you have here

is a model which mimics disease in another species. I think that the actual number of organisms doesn't make all that much difference.

It was interesting to see your data, which had to do with the relative physical clearance in the bacterial system and in the mycoplasma system, which was striking. It seemed to me that there could be several mechanisms that might explain that. One is that for some reason or another, the mycoplasmas were not going down into the deep lung. Most of them were being implanted on the ciliated epithelium, therefore they would disappear faster. Would it be explained by the difference in which the macrophages handled these organisms? We know that the phagocyte handles bacteria very quickly and efficiently. What is the status of the phagocytosis of mycoplasmas by macrophages?

DR. POWELL: Both your points are well taken. The first point was raised by Dr. Jakab, and we need to prove that the particle size of our aerosol gets to the alveolar level. We know that radioactivity is evenly distributed through the whole lung. That doesn't disprove that particles may be only going to the bronchiolar level. We've got to prove that they, in fact, reach the alveoli, which we're attempting to do. In terms of the macrophage, the only data that exist are the data that I showed you, and that's been corroborated by other people. Dr. Bredt in Germany has similarly shown that M. pneumoniae as well as other mycoplasma species are not rapidly phagocytized, despite not having a capsule or a cell wall. The mechanism by which they tend to avoid this phagocytosis has not been delineated, but clearly once they're opsonized with a specific antibody or complement they are engulfed.

Experimental Models for Study of Common Respiratory Viruses

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Many epidemiologic investigations have shown that there is excess mortality (10) and respiratory disease morbidity (16) in areas of high atmospheric pollution. These adverse health effects can be categorized in part on the basis of the toxic substance(s) involved; however, the mechanisms through which the pollutants exert their effects are poorly understood. While elimination of cause in a cause-and-effect relationship can be curative, this is not always possible or practical. A more complete understanding of the effect limb can suggest other approaches to modulate the end result of the cause-and-effect equation for benefit of the human host.

It is recognized that infections with common viruses are responsible for the vast majority of acute respiratory diseases. The viral agents involved are similar in nature and distribution in areas of both low and high atmospheric pollution. It follows, therefore, that an interactive effect of toxic exposure and infectious agent may be involved in the excess respiratory disease morbidity that has been described in polluted areas. Research on this problem has been limited by the ethical/moral constraints of human experimentation, and further by lack of clear indications for approaches to this issue. Use of experimental models of the common respiratory virus infections could provide the necessary foundation, by suggesting hypotheses to test in humans.

The purpose of this presentation is to review information concerning the development of practical animal models for important human respiratory viral infections. Following a brief discussion of the relative importance of different respiratory viruses, four examples of experimental model systems will

be described. In the context of this discussion consideration will be given to study parameters which could be meaningful for examination of the interactive effects of viruses and toxic substances.

IMPORTANT HUMAN RESPIRATORY VIRUSES

While the list of viruses that can infect the human respiratory tract is extensive, a relatively small number constitute those that are encountered most frequently (Table 1) (9). The relative importance of the different viruses varies according to host age and other epidemiologic factors. In infancy, respiratory syncytial virus (RSV) is of paramount importance and is the principal etiologic agent of epidemic bronchiolitis. Natural immunity to RSV appears to be minimal, and annual reinfections are frequent during the first years of life (8). Parainfluenza type 3 virus (P3) is another common cause of bronchiolitis; infections with this agent are seen in both epidemic and endemic patterns (7). Among pre-school children, parainfluenza type 1 (and type 2 to a lesser extent) virus infections are related to the occurrence of epidemic croup. The influenza viruses assume relatively greater importance as causes of respiratory disease morbidity in older children and adults. Experimental models for investigation of influenza virus infections have been available for many years, and will not be considered further in this presentation. Although outside the scope of this discussion, it should be noted that Mycoplasma pneumoniae infections are a very common cause of tracheobronchitis and pneumonia in older children and young adults. Experimental models of mycoplasma infection are described elsewhere in this symposium (D. A. Powell, P. C. Hu, A. M. Collier). Adenovirus respiratory diseases occur at all ages, although the types involved differ: in children, types 1, 2, 5, and 6 are encountered most frequently; in adults, types 3, 4, and 7 are seen particularly as causes of acute respiratory disease (ARD) of military recruits. Rhinoviruses assume importance as agents of significant respiratory disease morbidity primarily in adults.

From the foregoing it is apparent that research related to the interactive effects of pollutants and respiratory viruses should be targeted on those agents having the greatest human health impact. Toward this end, information will be summarized on experimental models of respiratory syncytial virus, parainfluenza type 3 virus, adenovirus, and rhinovirus infections.

MODELS OF RESPIRATORY SYNCYTIAL VIRUS INFECTIONS

Respiratory syncytial virus is an enveloped RNA-containing virus sharing some biologic properties of the paramyxovirus group. The agent is difficult to handle in the laboratory because of its lability and the limited range of susceptible cell culture types available for isolation and propagation of the virus. The natural host range includes at least the higher primate and bovine species,

Table 1. Non-bacterial Agents of Importance
in Human Respiratory Diseases

Agent	Host	Disease syndrome
Respiratory syncytial virus	Infants Children, adults	Bronchiolitis, pneumonia Rhinitis
Parainfluenza viruses		
Type 3	Infants Children, adults	Bronchiolitis, pneumonia Rhinitis
Types 1, 2	Infants, children Adults	Croup Rhinitis
<u>Mycoplasma pneumoniae</u>	Older children, adults	Tracheobronchitis, pneumonia
Influenza viruses	Children, adults Adults	Influenza Pneumonia
Adenoviruses		
Types 1, 2, 5, 6	Infants	Rhinitis, bronchiolitis, pneumonia
Types 3, 4, 7, 14, 21	Adults	ARD, pneumonia
Rhinoviruses	Adults	Rhinitis

in addition to humans. Initial work in search of animal models for RSV had only limited success (4). It was found that infection could be established in ferrets, although disease was limited to the nasal passages; hamsters and guinea pigs showed little evidence of disease manifestations after infection, although immune responses were measured. More recently, further studies of ferrets of different ages and of the cotton rat have yielded promising results.

The Ferret-RSV Model

Coates and Chanock first demonstrated susceptibility of the ferret to RSV in a search for animal models involving several other species (4). After intranasal inoculation, ferrets yielded virus from their tracheas for four days and

nasal turbinates for one week. Maximum viral replication occurred within three to four days, and the animals demonstrated brisk serologic responses in three to four weeks by the neutralization and complement fixation techniques. Histopathologic changes were limited to the nasal passages; after one week there was destruction of the ciliated epithelium, formation of multinucleated cells, and appearance of intracytoplasmic inclusions. Healing took place in 30 days, but submucosal cellular hyperplasia and irregularities in cartilage were seen as residua. Little use was made of this model subsequently due to the lack of significant pulmonary involvement.

In 1976, Prince and Porter (14) reexamined the possible use of ferrets for experimental RSV infections. They employed animals of various ages and reproduced the findings described above in adult animals. Moreover, it was found that virus replicated in the lungs of infant ferrets and that this event was age-dependent, disappearing by age four weeks. Viral antigen was demonstrated by immunofluorescence in the surface epithelium of the nose, and in alveolar cells within the lungs. Lack of bronchiolar involvement makes the infant ferret model an imperfect human counterpart; however, the age-dependent organ localization mimics the lower respiratory tract involvement with RSV seen in infants, and the upper respiratory tract disease of older children and adults. The model thus has usefulness for long-term studies and for examining the determinants of virus host-cell tropism.

The Cotton Rat-RSV Model

In 1971, Dreizen and co-workers (6) explored use of the common cotton rat (*Sigmodon hispidus*) as an experimental host for RSV infection. In contrast to the ferret, RSV replicated to high titer throughout the respiratory tract of the cotton rat. The time course of viral replication was similar to that seen in the ferret nasal turbinates, with maximal production around the fourth day following inoculation. Pathologic changes of rhinitis, bronchitis, and bronchiolitis were present which prominently involved the surface epithelium.

Recently Prince and co-workers (13) confirmed Dreizen's findings and added new information about the cotton rat model. It was found that age of the animal had no influence on susceptibility of the lung to RSV, unlike the ferret. Ciliated epithelial cells showed morphologic evidence of injury, with changes including cytoplasmic ballooning, loss of cilia, nuclear contraction, and cellular desquamation. Epithelial injury was most prominent in the nasal turbinates, but was present also in bronchioles. Viral antigen was present throughout the nasal epithelium as shown by immunofluorescence; additionally, antigen was identified in bronchial and bronchiolar epithelium, but not in the trachea.

Although information on human lung pathology in RSV disease is not extensive (5), the cotton rat observations appear to supply a reasonable counterpart in terms of the areas involved in the respiratory tract. Autopsy material

reveals a component of interstitial pneumonia and extensive peribronchiolar round cell infiltration, which has not been characteristic of the cotton rat lesions. However, the microbiologic similarities and host cell specificity provide a basis for many kinds of meaningful experiments; the increased volume of the bronchiolar epithelium may even compromise flow through small airways, making pathophysiological studies possible. Together, the cotton rat and the infant ferret provide the basic tools required to initiate an understanding of the pathogenesis of RSV disease. Unfortunately, cotton rats are not available commercially at the present time.

Other RSV Models

The initial recovery of RSV was from a chimpanzee with rhinitis; for a time the virus was called the "chimpanzee coryza agent." Apparently this animal is naturally infected with RSV and thus could serve as an experimental host. However, usefulness of the model is limited by cost and the difficulties attending studies with large primates. Limited experience also suggests that chimpanzees do not have pulmonary disease after inoculation with RSV. Sero-logic surveys have shown that Cebus monkeys regularly possess RSV antibodies, suggesting another animal which experiences natural disease with the virus (1).

Recently, a bovine RSV has been described, and experimental infection of calves is reported (11). Inoculated animals develop fever, serous rhinitis, peribronchiolitis, and pneumonia. While the calf could be valuable for certain kinds of studies, there are practical limitations for extensive laboratory investigations with animals of this size. Bovine antiserum against RSV is available commercially (Burroughs Wellcome, Ltd.) and has been used successfully for rapid immunofluorescence diagnosis of human infections; this suggests close similarity or identity of the human and bovine viruses.

MODELS OF PARAINFLUENZA TYPE 3 VIRUS INFECTIONS

As the name implies, P3 virus resembles influenza virus in some features; notable is possession of a hemagglutinin and neuraminidase, but these do not show the antigenic diversity of influenza virus. The virus grows readily in a number of cell culture types commonly used in the diagnostic laboratory. It can be recognized by characteristic cytopathic effects or by adsorption of erythrocytes, and can be identified by hemadsorption inhibition with type-specific antiserum. The natural host range includes man, guinea pigs, and monkeys; a similar but antigenically distinct virus is the etiologic agent of shipping fever in cattle and is widespread among many cloven-hoofed species. Practical experimental models to be discussed are the Syrian hamster and the guinea pig.

The Hamster P3 Model

Experimental P3 virus infections using hamsters were described in detail by Buthala and Soret (3), who examined virologic, serologic, and pathologic parameters. In initiating these studies, they found variable susceptibility to infection of animals from different sources. This variability was unrelated to age, sex, or weight of the animals and was thought to be due to partial immunity. Although P3 virus is not known to cause natural disease in the hamster, it is possible that human to hamster transmission occurs, or that they have endemic infection with a related agent such as Sendai virus (murine parainfluenza type 1). In any event, source of supply is a critical matter requiring careful control and monitoring.

Optimal infection of the hamster with P3 was achieved with intranasal inoculation of cell culture-passaged material. Virus replication reached a peak in the lung at three days, clearing spontaneously by one week. Pathologic changes were maximal at five to seven days, resolving by two weeks. Necrotizing nasal lesions were observed first, followed by extension of changes into the lower respiratory tract. The airway epithelium became hyperplastic with formation of multinucleated cells. Peribronchial and perivascular leukocytic infiltration was pronounced, but alveolar involvement was seen infrequently. Intraluminal exudates of leukocytes and desquamated epithelial cells were noted. Infected animals developed a brisk serologic response detectable with the complement fixation method which rose between the first and second weeks post-inoculation. The pattern of changes which accompanies P3 infection of the hamster is reproducible with regularity in susceptible animals. There are close similarities between the pathologic findings and information concerning P3 bronchiolitis and pneumonia in humans (5). Until animal models of RSV infections are developed further, the P3 hamster model can serve as a good system for studies on the pathogenesis of acute viral bronchiolitis.

The Guinea Pig P3 Model

Guinea pigs are known to be susceptible to P3 infection, and have been used extensively as donors of antiserum for studies on interrelationships of the paramyxoviruses. Details of the experimental disease produced in this animal have not been described, but unpublished observations (15) indicate a virologic and pathologic course very similar to that in the hamster. The guinea pig offers several advantages over the hamster for use as an experimental model. Many technical manipulations are done more readily in the guinea pig because of its size. Commercial reagents are available for immunologic studies. Also, the animals are highly sensitive to many pharmacologic agents, which could make them good models for pulmonary physiologic changes in consequence of infection. As with the hamster, guinea pigs are commonly immune to P3 which

probably reflects a combination of their sensitivity to infection and the ubiquitous distribution of the virus in nature. It is possible to produce colonies free of P3 experience, and methods for this purpose have been reported (17).

MODELS OF ADENOVIRUS AND RHINOVIRUS INFECTIONS

Although adenoviruses cause less respiratory disease morbidity than some other agents that have been discussed, they were among the first respiratory viruses discovered and have been studied extensively. These DNA-containing viruses constitute a large family of specific serotypes whose natural hosts include man, monkeys, cattle, rodents, dogs, and chickens. Generally, there is species specificity between host and given serotypes such that experimental models are not readily available. Considering the types most frequently encountered in humans (see Table 1), hamsters, dogs, and rabbits may be infected but show no overt signs of illness. Latent infections may become established in hamsters; several serotypes are carcinogenic, resulting in sarcomas at the site of inoculation. Pulmonary lesions can be produced in pathogen-free, colostrum-deprived piglets with serotypes 1, 2, 5, and 6 (2). A fatal disease can be produced in newborn hamsters with serotype 5 (12).

The rhinoviruses are etiblogic agents of many common colds, especially among adults, and thus are important for study in the context of this symposium. These RNA-containing viruses demonstrate a multiplicity of serotypes, which complicates their identification and the performance of serologic testing. Special conditions are required for rhinovirus isolation, including selected cell cultures, and low incubation temperature and medium pH relative to other groups of respiratory viruses. The host range of these viruses appears limited (9). Cattle experience natural infection with biologically similar agents; chimpanzees and gibbons can be infected with some of the human serotypes. Experimental infection of a variety of common laboratory animals has been unsuccessful.

Progress in the development of techniques to maintain organ or specialized cell cultures (see A. M. Collier, this symposium) provides the potential for use of human cells as hosts or the adenoviruses and rhinoviruses of interest. These model systems provide easily controlled circumstances for detailed study of the host-parasite interaction, but obviously lack the ability to respond like the intact host. Nevertheless, simplified models could prove very useful in dissecting the cellular basis of the interactive effects of toxic substances and infectious agents.

RESEARCH STRATEGIES USING EXPERIMENTAL MODELS OF RESPIRATORY VIRUS INFECTIONS

The foregoing discussion has emphasized the development of animal models of the more common human respiratory virus infections. Consideration will now be given to meaningful ways in which these model systems might be used to study the interactive effects between the viruses and pollutants of interest. This symposium has addressed a wide variety of host defense mechanisms and pharmacologic reactions, all of which could be evaluated in the experimental infections described.

Information accumulating on the pathogenicity of different infectious agents suggests many mechanisms by which host defenses are bypassed. Depending upon the target cell for a given agent within the respiratory tract, the microbial offense can relate primarily to evasion of muco-ciliary clearance, avoidance of phagocytosis, exertion of immunosuppressive effects, and the like. It is probable that effects of toxic substances have a similar specificity; accordingly, the most appropriate study parameters must be chosen considering both infectious agent and pollutant. Concerning respiratory viruses, the microbiologic, immunologic, pathologic, and physiologic dimensions of the experimental models could be explored to determine the superimposed effects of toxic exposures.

The virologic aspects of the experimental models have been defined in terms of the time course of infection, amount of viral replication, and principal host cell types involved. Adverse effects of toxins might be reflected in longer viral persistence, higher titers, and more extensive involvement of host cells. The role of toxins in these effects could include alterations in host cell receptor sites for the viruses, and in the immune and phagocytic mechanisms required for control and elimination of infection.

Studies on the immunologic aspects of the experimental infections are facilitated by the information developed in recent years concerning the respiratory tract. The presence of virus-specific secretory IgA in the upper respiratory tract correlates well with the presence of protective immunity. Antibodies of other classes can be produced locally by the bronchus-associated lymphoid tissue, and probably function in neutralization of free virus, prevention of host cell attachment, or opsonization for phagocytosis of infected cell debris. Since intracellular virus is protected from the effects of antibody, another important mechanism concerns cellular immunity provided by thymus-derived lymphocytes. Immune cells which are specific for both virus and host cell type are capable of lysing virus-infected cells, thereby contributing to control of the infection. The immune mechanisms described are operative in or on the respiratory mucosal surface, and thus may be particularly vulnerable to the effects of atmospheric pollutants.

The pathology of experimental infections could be altered in a variety of ways by the superimposed effects of toxic exposure. Attention could be

focused on the degree, extent, and persistence of histopathologic changes in the lung. The models described in this presentation are self-limited diseases, so that the occurrence of mortality would provide meaningful information. Sublethal effects in regard to the healing and repair processes also should receive attention, since the interaction of infection and toxin might produce long-term sequelae.

Lastly, physiologic studies are important to accomplish in the experimental model systems. Correlates are needed for the pathophysiologic changes in humans which have been important indicators of the health effects of pollutants. Methods are available to perform respiratory physiologic studies in small laboratory animals, but thus far application of these procedures to models of infectious diseases has been limited. Some of the anatomic considerations that have been described suggest that pathophysiologic changes could be a very sensitive measure of the modulation of disease by toxic exposure.

In summary, several practical laboratory animal models for common human respiratory virus infections have been described. These models lend themselves to studies on the interactive effects of pollution and infection, thus addressing an important human health problem. Although findings from such studies cannot be extrapolated directly to the human situation, they can serve as indicators of health effects which should be sought and of appropriate methods of study that could be employed.

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Session V:

**Regulation of Mucus Secretion
and Cellular Differentiation**

Mucous Membrane of Respiratory Epithelium

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Although there are animal models in which the airway damage is associated with necrosis and sloughing of epithelium, this report concentrates on animal models in which the nature and dose of the damaging agent are such that the epithelium remains intact. Such models are especially relevant to understanding the effect of a relatively low dose of irritants delivered in either acute or chronic exposure. It is a remodeling of the cell population of the normal intact epithelium that occurs, one that is particularly related to mucus hypersecretion, the most obvious single feature of the response and almost certainly the most important functionally (24, 30).

Even when the injury is serious enough to cause ulceration, we have shown healing to occur while the administration of the irritant continues and, in the new epithelium, remodeling is seen similar to that discussed below (31). Our most recent studies show that changes that we identify with chronic irritation occur quickly, much more quickly than previously suspected (11, 18).

In such studies it is essential to use animals with "clean" lungs (1, 2). The normal human airways are sterile and even with mucus hypersecretion this state often continues. We find that histological criteria are more sensitive to determine satisfactory cleanness than bacteriological or viral studies. Lymphocyte infiltration may be of an unacceptable degree even when the lungs are sterile.

The mixed nature of the mucus or total bronchial liquid, and the variety of epithelial cells responsible for the special mucus or epithelial glycoproteins (9, 10, 33) call for a multidisciplinary approach. Animal models or

in vivo studies with which we are particularly concerned here can be complemented by in vitro studies of the tissue cells (3, 37, 38). Histochemical studies (11, 16, 17, 21, 22, 23, 35) are complemented by biochemical analysis of total secretion, both its bronchial and serum components (7, 20, 25, 26, 27). These in turn need to be correlated with rheological behavior (19, 25, 27).

CELLS OF THE RESPIRATORY EPITHELIUM

Within the airway epithelial lining eight epithelial cell types have been identified (9, 10) (Fig. 1). All cells touch the basement membrane; only two do not reach the lumen--the basal and Kultschitsky cells. The serous, Clara, and mucous are the secretory cell types. The undifferentiated or intermediate cell and the ciliated and brush cells are not secretory. "Mucous" is the term we now prefer to goblet cell, since we can define it by reference to the nature of the secretory granules, whereas "goblet" refers essentially to the amount and shape of the secretory mass which resembles a chalice or goblet. The serous and Clara cells are characterized by electron dense and discrete granules: the Clara cell has a considerable amount of smooth endoplasmic reticulum and its apex often bulges above the tight junctions with neighboring cells. Two mesenchymal cells--the globule leukocyte and the lymphocyte--are found.

Reconstruction of the human submucosal gland reveals a duct about 1 mm long, lined by the duct cell--a tall cell, packed with mitochondria and reminiscent of the striated duct of the salivary gland but lacking the striations (Fig. 2) (29). The tubules are lined by mucous or serous cells--the "serous" regions being always distal to a mucous region, i.e., farther from airway lumen, and arising either as a lateral pouch or from the distal ends of a mucous tubule (28).

Nerve fibers are found within the airway epithelium (8). In the rat, they are non-myelinated and only in the extrapulmonary airways. They are numerous, particularly in the young animals, and are closely associated with basal secretory and ciliated cells. The nature of the vesicles they contain suggests that some are sensory, some are motor, and of the latter, some are adrenergic and some are cholinergic. Nerve fibers are found within the basement membrane of the submucosal glands. Myoepithelial cells and "clear" cells, probably immunoblasts, are also found in the secretory tubules of the gland.

In the rat, mitotic activity represents turnover of the airway epithelium that is, in some regions, as fast as 10 days and in others 250 days. This range takes into account all regions, males and females, and three ages (1) (Fig. 3).

NORMAL HOMEOSTASIS

Under basal conditions homeostasis of the airway epithelium is maintained with regard to several of its features, the normal pattern varying from region to region. The nature of the control mechanisms is not understood. Homeostasis

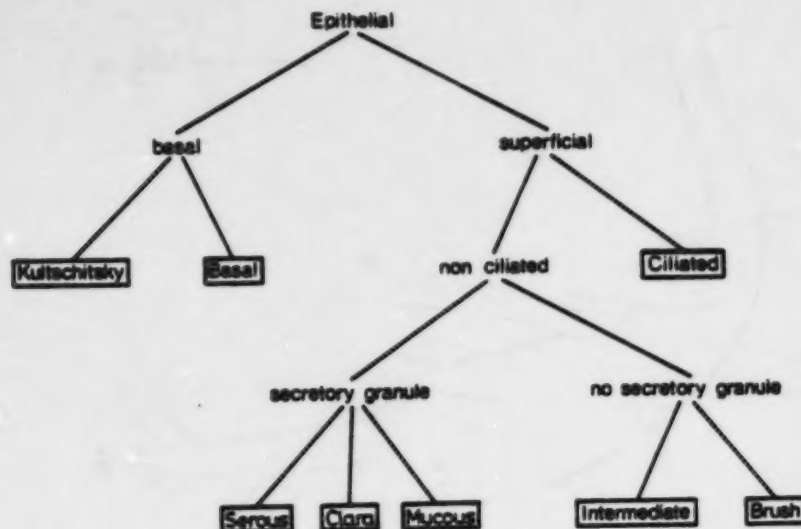


Figure 1. Eight epithelial cell types present in rat airway epithelium.

is maintained with respect to the cell types present. For example, in the trachea the basal cell is numerous, making up about 20% of the cell population, whereas it is virtually absent in peripheral airways. The Clara cell, the secretory cell of small airways, is not seen in the large central ones. The proportions of the cells that are present vary, the ciliated cell being about 1 in 5 centrally and 2 in 3 peripherally.

At a given airway level, the pattern of glycoprotein within the secretory cells varies (11, 13, 15, 21). The epithelial glycoprotein is either neutral or acid (21-23): it is acid either because of sialylation or sulfation of the glycoprotein, and the sialic acid may or may not be susceptible to digestion by the enzyme sialidase (Fig. 4). Sometimes within a single granule only one type of glycoprotein is identified or there may be a mixture. A cell can be characterized by its predominant granule type and by the "amount" of secretion, i.e., by the number of granules it includes. Regional differences in the normal pattern of glycoprotein distribution are also seen.

Cell division is another feature having a basal level of activity (1). The oldest group of animals we have studied did not show a regional pattern, in that the percentage of cells incorporating ^3H -thymidine was similar at all airway levels. Since concentration of cells is less in peripheral airways, this means a slower replacement per unit area in small than in large airways. In the young animals, in males more than in females, mitotic activity is more marked in trachea than in peripheral airways. This sex difference was apparent in the young animals although male and female animals were gaining weight at the same rate.

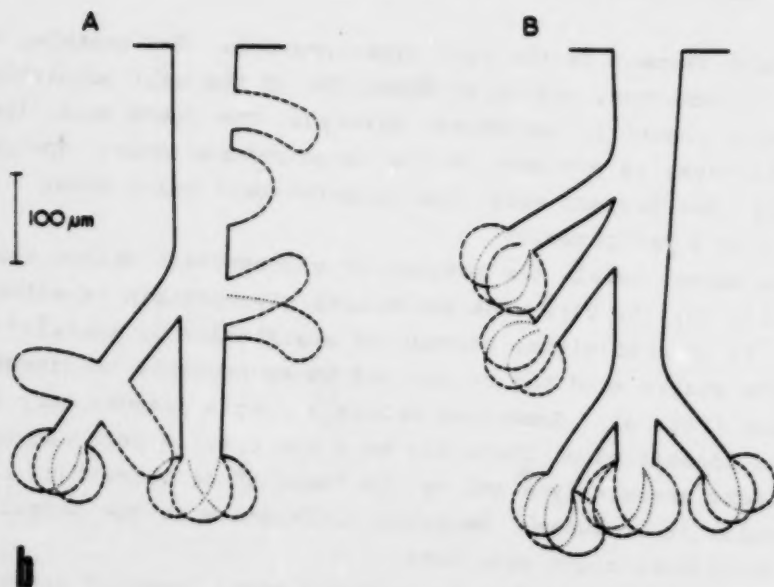
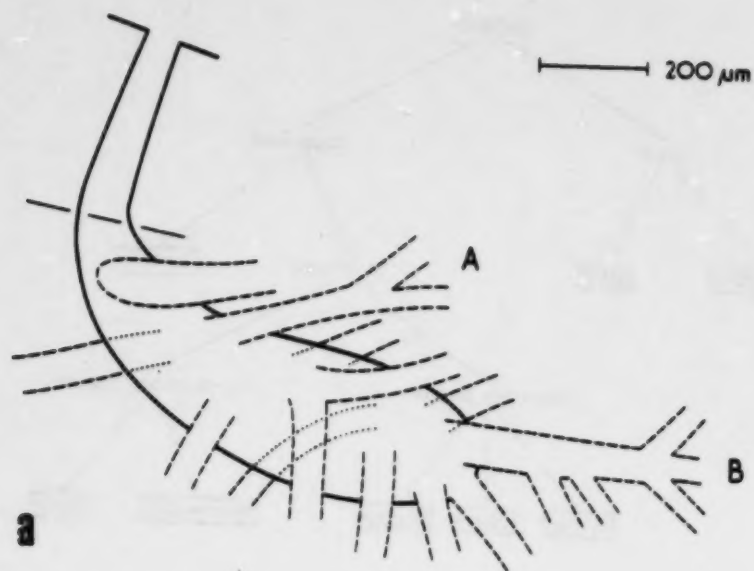


Figure 2, a and b. Reconstruction of human bronchial gland, drawn to scale, shows collecting duct with tubules arising from it. The branching patterns of two tubules arising from the duct are shown (A and B). Mucous cells (continuous lines), serous cells (broken lines). Secretion from the serous cell thus passes over the mucous cell before reaching the collecting duct. Reprinted with permission (29).

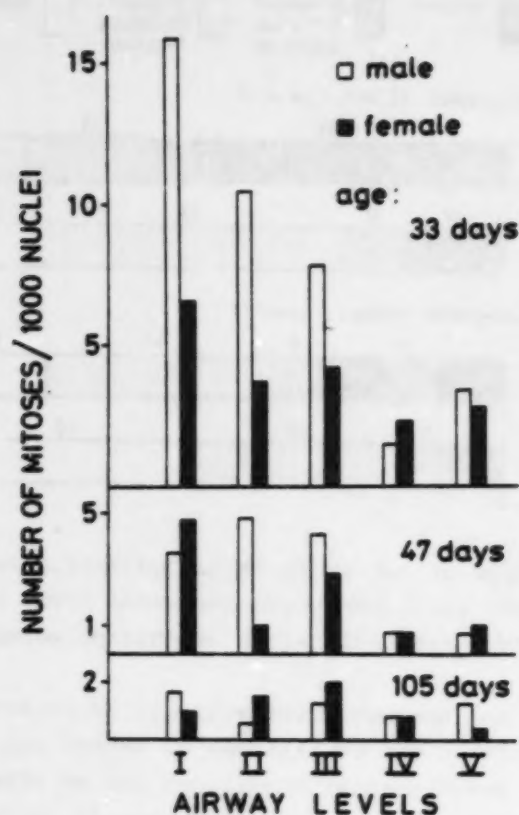


Figure 3. Mitotic index (number of mitotic nuclei per 1000 nuclei) in male and female rats of three age groups and at five airway levels. Levels I-III: proximal, mid, and distal trachea; Level IV: main intrapulmonary axial pathway; Level V: distal airway. Reprinted with permission (1).

Homeostasis is maintained, on a regional basis, with respect to types of cells present, their proportion, their rate of mitosis, and the pattern of intracellular glycoprotein identified histochemically.

ANALYSIS OF MUCUS SECRETION, CELLULAR DIFFERENTIATION, AND THEIR CONTROL

The mucus glycoprotein comes from several cell types both within the sub-mucosal glands and the surface epithelium; the mucus is then mixed with serum components. The structure of the airway wall changes with age and disease. Because of its complexity, to understand airway secretion and its control we have used a multidisciplinary approach in its study. Tissue studies of the normal human airway and its modification in hypersecretory states offer an important reference. In organ culture systems of human airway, the behavior of

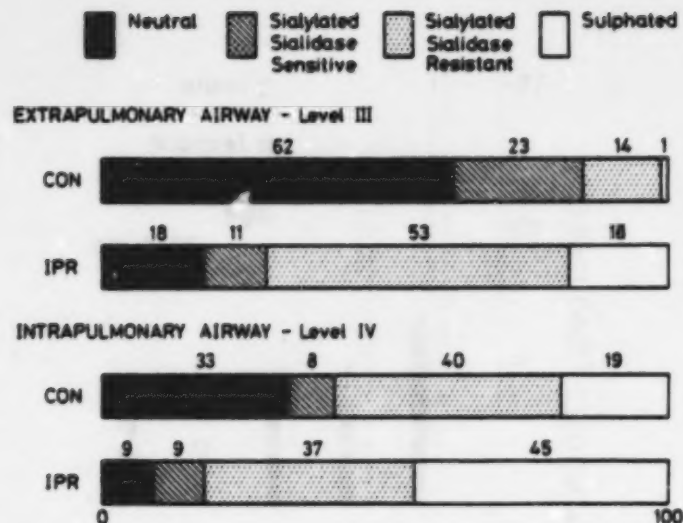


Figure 4. Percentage of secretory cells containing four types of glycoproteins in control (CON) and isoproterenol-treated (IPR) rats. Level III: distal trachea; Level IV: proximal region of intrapulmonary axial pathway.

the various cell types and the way their activity is controlled can be explored (5, 6). The total secretion can be analyzed as sputum and as material collected in organ culture (3). Environmental conditions can be altered in animal models. By organ culture of tissue obtained from such animals in vivo and in vitro studies can be correlated (4, 34).

Our more recent organ culture studies of human epithelium show that secretory cells of the surface epithelium, at least of the large airways, unlike the submucosal glands, are not stimulated to secrete by acetylcholine or its analogues. ³H-threonine transport into mucous and serous cells is largely ouabain-sensitive, that of ³H-glucose only partially so. Post-pulse addition of several agents has established that precursor uptake and glycoprotein synthesis and discharge by mucous and serous cells are not coupled events (6). In a series of experiments the effects of colchicine and cytochalasin B have been followed (5). Neither agent influences the basal rate of secretion of either mucous or serous cells. When incubated with the tissue and radioactive precursor, both reduce acetylcholine-stimulated secretion. It seems that the cytochalasin B-sensitive filament system must be intact for the discharge effect of a cholinergic agent.

RESETTING THE HOMEOSTATIC MECHANISM

The homeostatic mechanism is reset to a different level by a variety of stimuli-irritants, infections, and drugs.

Irritants

The biological response of the epithelium is illustrated first by its reaction to tobacco smoke (4, 14, 15) with or without the addition of an anti-inflammatory agent (Fig. 5). Phenylmethyloxadiazole (PMO) is the agent illustrated although similar results have been produced by phenylbutazone. Rats were exposed to a smoke from cigarettes, delivered from a Wright auto-smoker over a period of four hours (Table 1). In the first experiment described, this 4-hour daily exposure was repeated for four days a week for six weeks. The tobacco produced an increase in the concentration of secretory cells and of ciliated cells at all airway levels. Phenylmethyloxadiazole prevented this in the extrapulmonary airway but had no such "protective" effect on intrapulmonary airways. Analysis of intracellular types of glycoprotein showed a shift in their pattern. In the trachea the shift is from a population of cells containing predominantly neutral glycoprotein to one that is acid-producing. This shift occurs whether or not PMO is administered, that is, whether or not there is an increase in secretory cell number. From these studies we know that this shift can be detected within 24 hours, making it the most sensitive marker of change in airway epithelium.

Tobacco also increases mitotic activity. A burst of mitosis is seen 24 hours after starting exposure to tobacco smoke; PMO gives partial protection against this increase. The increase in secretory cells, particularly mucous cells, arises, in part, from conversion of undifferentiated cells to secretory cells, from new cells that appear as the result of mitosis, and within the secretory cell population by interconversion of serous or Clara cells to mucous cells. The interconversion is apparent from cell counts and from identification of intermediate cell forms. Electron dense granules become mixed with electron lucent granules until, finally, all are electron lucent and "confluent." By light microscopy it is possible to identify an increase of Alcian Blue staining granules. When a cell switches to secrete acid glycoprotein, the AB positive granules appear first at the apex of the cell. The "switch-on" of enzyme responsible for the attachment of sialic acid seems to start near the cell apex after the secretion is already packaged in its granules. The shift is first to sialylation and then to sulfation. When the latter occurs, the sulfated granules also appear first at the apex, with sialylated granules being present nearer the nucleus.

In studies of shorter exposure time, it emerged that this lability of the cell population, particularly of its secretory cells, is apparent after only one exposure to tobacco smoke (18) (Fig. 6). In this experiment the response to tobacco smoke was monitored at intervals up to 14 days. Animals were sacrificed 20 hours after the last exposure. Extrapulmonary and intrapulmonary airways showed different patterns of response. In the case of the trachea, one period of exposure to tobacco produced a "discharge" effect so that the total

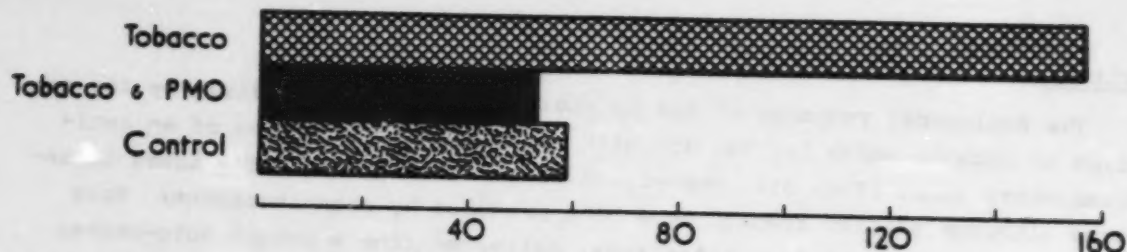


Figure 5. Mean number of secretory cells in 6 mm of rat tracheal epithelium after inhalation of tobacco smoke (tobacco), alone or with phenylmethyloxadiazole (tobacco + PMO), or after air alone (control).

Table 1. Mean Number of Secretory Cells in 3 mm of Epithelium in Control Rats and After a Single 4-Hour Period of Inhalation of Tobacco Smoke

		Level	Control	Tobacco
Extrapulmonary airways	{	I	34 $\pm$ 8.2	5 $\pm$ 1.9**
		II	41 $\pm$ 8.1	7 $\pm$ 0.9**
		III	64 $\pm$ 6.5	53 $\pm$ 4.6
Intrapulmonary airways	{	IV	27 $\pm$ 6.4	64 $\pm$ 7.4*
		V	6 $\pm$ 2.8	17 $\pm$ 5.3
		VI	28 $\pm$ 6.4	29 $\pm$ 9.7
		VII	6 $\pm$ 2.0	7 $\pm$ 0.2

*p < 0.05, **p < 0.01.

secretory cell number was reduced. One exposure produced tolerance to the discharge effect so that after two days of exposure the secretory cells had increased significantly above control values. In the intrapulmonary airways no discharge effect was seen: the one 4-hour exposure period significantly increased the secretory cell number above control values. Some further increase developed during the second week, when the concentration of secretory cells was virtually the same as that seen after six weeks of exposure. (This was a separate experiment.)

In the trachea, tolerance to exposure is quickly lost. One day of rest from exposure restores tolerance so that the next exposure produces a discharge effect. Adaptation to the environment is rapid and in a short time the maximal effect is produced. Although tolerance is quickly achieved, it also seems to

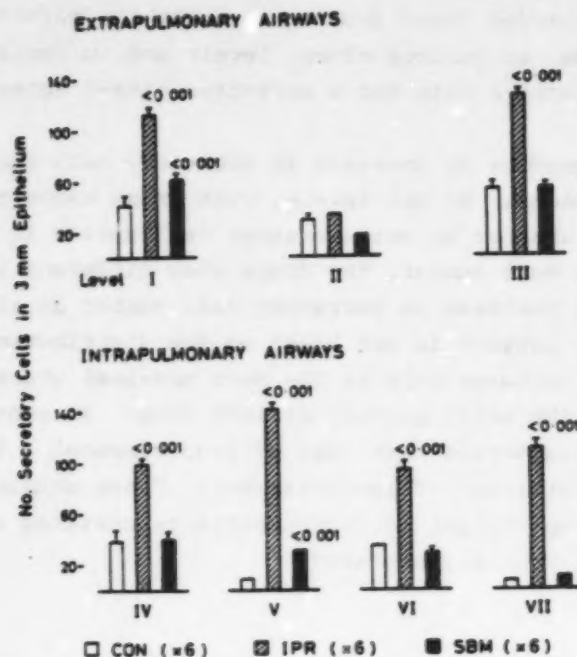


Figure 6. Mean number of secretory cells in 3 mm of airway epithelium of control (CON), isoproterenol-treated (IPR), or salbutamol-treated (SBM) rats at seven levels. Levels I-III: proximal, mid, and distal trachea with main bronchi; Levels IV and V: proximal and distal region of main intrapulmonary axial pathway; Levels VI and VII: lateral branches. Reprinted from Fed Proc 38:191-196, 1976

be quickly lost. The homeostasis mechanism is reset for each of its features, secretory cell type and population, mitotic activity, and glycoprotein pattern; a regional pattern is still apparent.

The anti-inflammatory agent PMO has modulated the response, offering protection from some aspects of the irritant but not from others; PMO given alone, by parenteral injection, caused increase in submucosal gland size. *In vitro* study of submucosal gland from these animals showed reduced precursor uptake, as well as decreased discharge. The animals that had received tobacco plus PMO showed normal secretory rate, whereas tobacco alone produced an increase in secretory rate.

Drugs, Beta-adrenergic Agents--Isoproterenol and Salbutamol

We have shown that isoproterenol causes hypertrophy of the bronchial submucosal gland and increases surface epithelial secretory cell number even under germ-free conditions (12, 32, 33, 39). The secretory cells producing acid glycoprotein are particularly increased in the surface epithelium. In a recent ex-

periment, we have extended these studies to study the effect of isoproterenol over shorter times and at various airway levels and to compare it with that of salbutamol (a nonselective beta and a selective beta-2 agonist, respectively) (Fig. 6).

The two drugs produce an increase in secretory cell number, but with striking regional differences. At all levels, both drugs cause a shift to acid glycoprotein production, whether or not secretory cell number is increased. With respect to secretory cell number, the drugs show different regional specificities. Isoproterenol causes increase in secretory cell number at all levels except the mid-trachea (so this pattern is not based on the distribution of nerves). Salbutamol produces an increase only at the most proximal tracheal level and the most distal part of the axial pathway of left lung. At each level the salbutamol effect is less marked than that of isoproterenol. The effect can be detected after one injection of isoproterenol. These studies also show that the lability of the epithelial cell population is striking and rapid, and the "maximal" response is quickly achieved.

RECOVERY

After administration of the drug was ceased, "recovery" was followed and here also major regional differences are seen (Fig. 7). In the trachea the secretory cell concentration returns virtually to normal within a day or so. In other regions differences persist. In the main bronchus, even 12 weeks later, secretory cell number has not returned to normal. At this level there is a stage when secretory granules are seen as ghost structures, and an irregular mixture of granules of acid and neutral glycoprotein is seen, different from the orderly way in which a "switch-on" to acid glycoprotein occurs. It seems that the airway does not become tolerant of the stimulus, the tissue does not "adapt" permanently since on withdrawal of the isoproterenol the airway reverts to normal. During recovery a greater variance is seen between individual animals in the same group than during adaptation to the drug. This is particularly true of surface epithelium. In the submucosal gland hypertrophy is also produced by isoproterenol, and has been shown to revert to normal within six weeks (12).

It seems that this pattern of response reflects regional differences in sensitivity, based perhaps on regional differences in the nature and concentration of receptors. Since these drugs mimic naturally occurring agents it could be that the adrenergic system, beta and probably alpha, is part of the normal control system. Either way, the use of the appropriate agonists and antagonists offers a way to modulate secretion, certainly experimentally and perhaps also clinically.

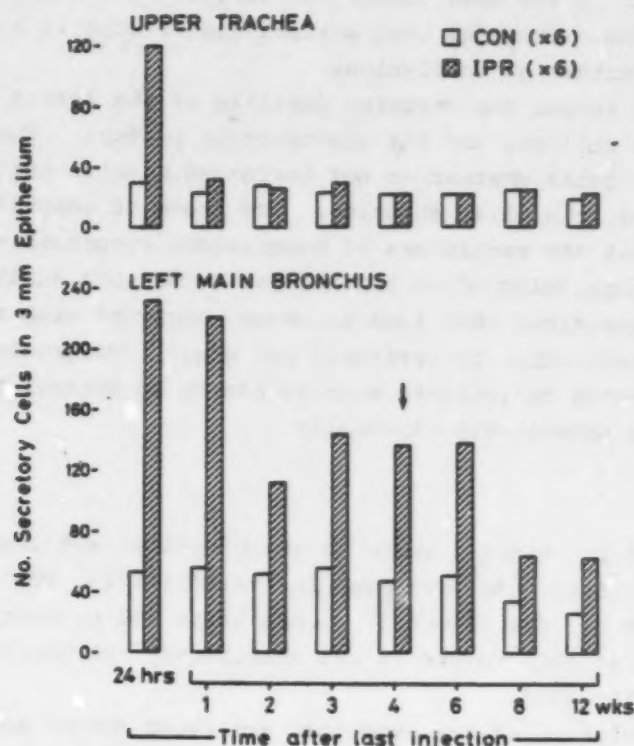


Figure 7. Mean number of secretory cells in 3 mm of epithelium in the upper trachea and left main bronchus of control (CON) and isoproterenol-treated (IPR) rats after single daily injections on 6 consecutive days of either normal saline (controls) or isoproterenol (10 mg/100 g body weight). Number of cells present 24 hours after the last injection and after periods of 1 to 12 weeks recovery.

Intra- and Extracellular Conditions

The histochemical studies of the glycoprotein within the cell are of special significance in showing the shift to increased acidification of the oligosaccharide side chains, with a relative increase in sulfated forms (Fig. 4) and, within the granules that are only sialylated, to those in which more of the sialic acid is resistant to sialidase. Understanding the composition and structure of bronchial mucus glycoproteins (as of other epithelial glycoproteins, e.g., gastric and cervical) is an actively expanding field, both as regards the polypeptide core and the oligosaccharide side chains (7, 27, 36).

The histochemical shift seen in states of hypersecretion is associated with the biochemical finding of increased concentration of both N-acetyl neuraminic acid (syn. sialic acid) and sulfate in the oligosaccharide fraction.

Sometimes both occur on the same chain, the largest chains are usually the most heavily sulfated. It seems that whereas sialic acid is a terminal sugar, sulfation permits further glycosylation.

Animal studies reveal the striking lability of the intact epithelium, both as regards its cell mix and its glycoprotein product. These adjustments occur in the airway cells whether or not increased mitotic activity is also part of the response to a given stimulus. The speed of adaptation and its nature raise questions about the mechanisms of homeostasis responsible for stability in such a complex population of cells that yet shows such striking regional variation. These questions then lead to those concerned with the basis of the resetting of this regulation by irritants and drugs. Understanding these changes can be expected to indicate what is needed to control the features of adaptation that are undesirable clinically.

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DISCUSSION

PARTICIPANT: Do you find that isoproterenol changes the nature of the airways in that it increased secretory cells of different types? For instance, where the Clara cells are, do you find new types of secretory cells or an increase of those types?

DR. REID: We find new types. The first thing I should say is that with tobacco, the increase in secretory cells was at the expense of the intermediate cells: both ciliated and secretory cells increased. With isoproterenol, the first counts indicate that it's only the secretory cells. When the cells change, Clara cells virtually disappear. Clara cells develop into cells that look just like mucous cells. You see the stage where even the smooth endoplasmic reticulum becomes less abundant. That's one feature of the cell. Electron dense granules develop, then a mixture of these and electron lucent granules is seen, and ultimately all become electron lucent.

DR. ADLER: With exposure to tobacco smoke, do you find a massive shift in the secretory serous cells to the mucous cells before you get an increase of electron dense granules in the cells?

DR. REID: Yes, because we can pick the increase at 24 hours, and the first wave of mitosis occurs at about 24 hours. It is clearly not as obvious because the dramatic increase of cell number occurs about two days in the tobacco group.

DR. NETTESHEIM: In the literature there is a lot of confusion about terms. Do I understand you correctly that you consider the goblet cells, secretory cells, and mucus-producing cells in the upper airways are cells of different functional states of the same cell type?

DR. REID: I would feel that at the moment. First of all, how we came to use the three names. The serous cell is electron dense with mainly rough ER. The Clara cell differs widely in different species, but for this purpose, we can pick the main feature as being electron dense granules. That is the basis for the three terms. At normal baseline, with homeostasis at work, those three cell types are fixed, because there are only certain cell types at a given

level of the airway, and much that these cells are "end" cells, as it were; they are fully differentiated, and do not change. Under conditions of irritation, by drugs, by infection, then you will get a shift of one cell type to another. To say "stages" would imply that a cell will automatically go through all its stages, and I don't think the cells always go through all their stages. If we talk about secretory cell forms, it might remind us that under conditions of irritation or drug treatment, you can convert one of those cell types to another. Does that seem reasonable?

DR. GOLDSTEIN: What is known about the physiological consequences of the various types of glycoprotein that might give some teleological reasons for these very interesting and rapid shifts?

DR. REID: Not a lot. The fetus produces only sulfated glycoprotein, not sialylated at all. It isn't until about four months after birth that the sialylation of the bronchial glycoprotein occurs. I'm talking about the human. One of the epithelial glycoproteins that we've been able to study, which is only sulfated, is of the fish, and it had very low viscosity. Those are facts. Now to be teleological and to make a hypothesis. It's quite clear that you don't want the normal bronchial tree to be blocked by thick secretions soon after birth. One could suggest that the sulfation is to keep viscosity low. In disease, for example, chronic bronchitis, the gland is hypertrophied. We know that both sialylation and sulfation increase, and viscosity increases as the concentration of the mucus arises.

So there is evidence that viscosity increases as sialylation and sulfation increase. The longer oligosaccharide side chains tend to have more sulfated, but sialic acid always comes in a terminal position. The sialic acid content of the glycoprotein increases in the bronchitic, compared with normal secretion from a normal airway--in particular when compared with prostaglandin F_{2a} stimulated secretion. It's difficult to be more precise than that.

New Methods Used To Investigate the Control of Mucus Secretion and Ion Transport in Airways

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Mucins secreted from submucosal glands and surface epithelial cells combine with water to form the respiratory tract secretions. The secretions with trapped inhaled particles are moved up the airway to the mouth by the sweeping action of the cilia. This clearance process may be controlled by neurotransmitters or mediators acting (a) on secretion of mucin from submucosal glands and from surface epithelial cells; (b) on the composition or consistency of mucus; (c) on the interaction of the cilia with the mucous layer; and (d) on the rate of ciliary beating. These controls may be altered by environmental toxins, infections, inflammatory responses, or abnormal biochemical pathways and cause disease.

Knowledge gained from previous studies of airway epithelium has recently been reviewed (25). In the past, studies of respiratory tract secretions in man depended on the collection of sputum (2, 17, 28) which is contaminated by saliva and nasal mucus, or the collection of samples of mucus secretion via a tracheostomy (28). Knowledge of the action of drugs and mediators on mucus secretion was inferred from measurements of respiratory tract secretions in animals (3, 4, 16) or from changes in volume in a fluid-filled segment of trachea (14). These older methods do not localize the source of the secretions to submucosal glands or surface epithelial cells; therefore, they cannot be used to examine the control of secretion.

In this review we describe new methods for studying the control of mucus secretions that we developed using preparations of trachea in vivo and in

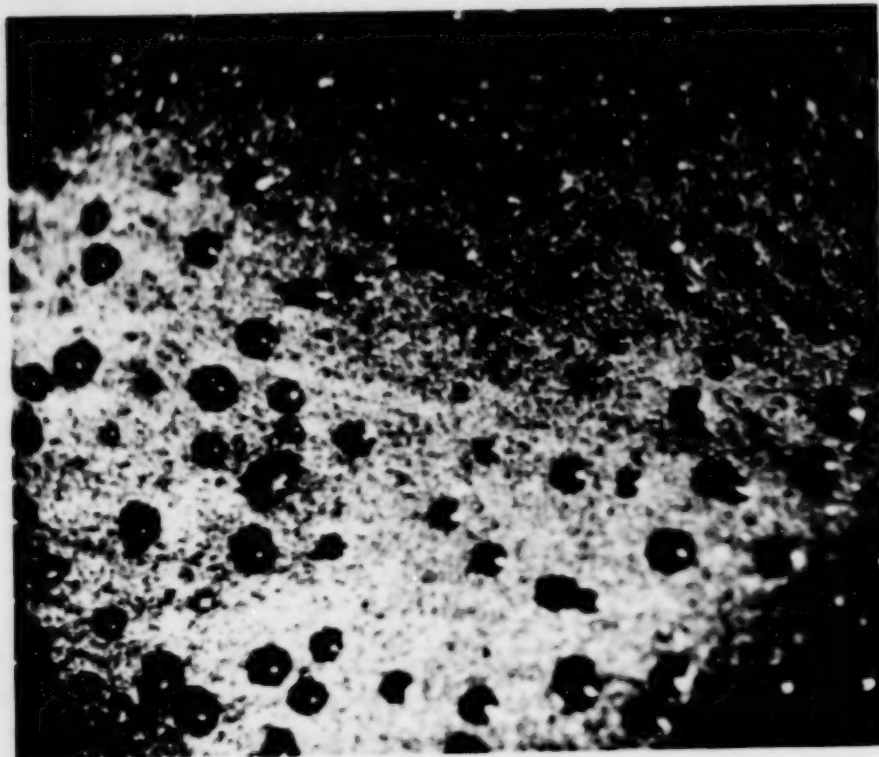


Figure 1. Photograph obtained through a dissecting microscope of hillocks on tantalum-coated canine tracheal epithelium produced by electrical stimulation of a superior laryngeal nerve. The tantalum prevented the normal dispersion of the secretions from the mucous glands. The hillocks, caused by accumulated secretions, averaged 0.2 mm in diameter.

vitro. Preliminary descriptions of some of these methods have been published previously (7, 9, 18, 22-24, 30). The methods provide direct evidence of secretion from the submucosal glands and allow us to study the functions of airway epithelium under well-controlled conditions.

IN VIVO TECHNIQUES

To study the control of secretion of individual mucous glands, we developed techniques that allow us to study the submucosal gland as a physiological subunit in vivo. We anesthetize dogs and ventilate the lungs artificially using a Harvard pump. Both vagus nerves are dissected free and placed on stimulating electrodes. We make an incision in the anterior midline of the upper two-thirds of the trachea and pull the cut edges apart

widely to expose the epithelial surface. A fine powder of inert metal (tantalum) is then sprayed onto the epithelial surface. Since the normal dispersion of secretions from the submucosal glands is prevented, elevations in the tantalum layer at the sites of the submucosal gland duct openings appear during secretory activity (Fig. 1). We call these elevations which are caused by submucosal gland secretion "hillocks." We have demonstrated that our new technique is suitable for neurophysiological studies. In each observation period, after the previously secreted mucus has been removed by wiping, the mucosal surface is coated with powdered tantalum and photographed through a dissecting microscope (magnification, 6X). We photograph the tantalum layer over the same area of epithelium at one-half minute intervals during baseline conditions and after stimulating either the superior laryngeal or recurrent laryngeal nerve electrically. The number of "hillocks" on each photograph is counted and divided by the area of epithelium to obtain the number of "hillocks" per square centimeter. These studies show that submucosal gland secretion is increased by electrical stimulation of the superior laryngeal and recurrent laryngeal nerves (7). We are able to prevent the secretomotor effect of stimulating the motor branches of the vagus nerve by atropine sulfate given intravenously or locally, indicating that the secretomotor effect involves postganglionic cholinergic pathways.

The measurement of the number of hillocks per square centimeter of tracheal epithelium does not provide evidence about secretion from individual submucosal glands; therefore we modified our photographic measuring system (10). Now we visualize the hillocks with a television camera attached to a dissecting microscope, record their images with a videotape recorder, and view the field during the experiment on a television monitor. A record of time is simultaneously displayed on the monitor and recorded on videotape. Thus, we make a continuous record of the secretion from individual submucosal glands; the rate of secretion can be estimated from the increase in the size of a hillock with time. We divide the observed field into six sections and measure the diameter of one round hillock in each section.

We measure the diameters of the hillocks using a split-video image measuring device (Instrumentation for Physiology and Medicine, San Diego). The images of the hillocks are played back from a videotape recorder onto the screen of a modified television monitor. The image is held on the screen by stopping the videotape recorder at any desired time. To measure the diameter of a hillock, we select the line in the video image which runs along the diameter of the hillock, offset electrically the upper part of the image of the hillock, and displace it from the lower part of the image of the hillock by the length of one diameter (Fig. 2). The distance moved, which equals the diameter of the hillock, is shown on a digital scale, which we convert to millimeters with a calibration factor. This measuring device

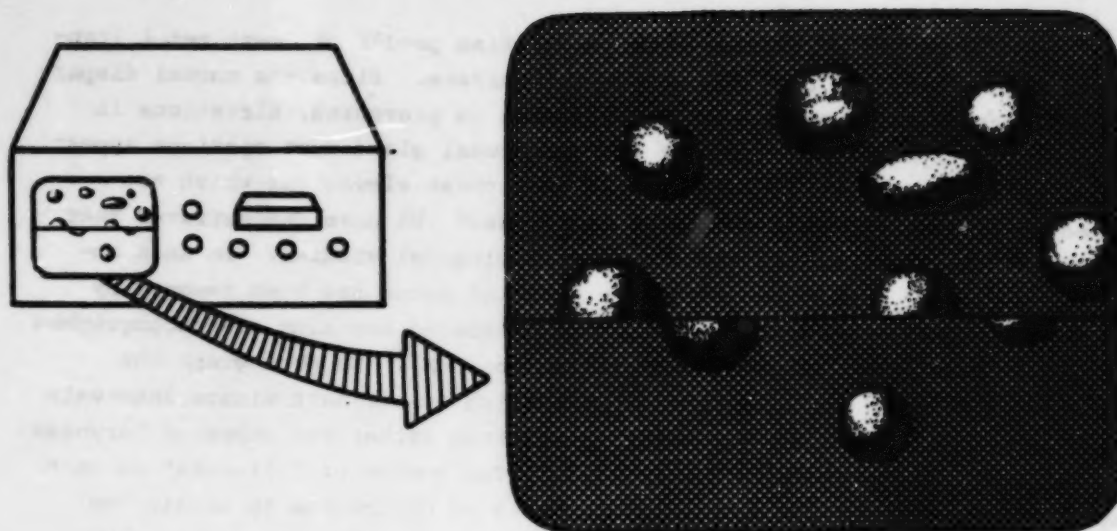


Figure 2. Enlargement of the screen of a video-image measuring monitor showing a picture of hillocks projected from a tape recorder. The image of one hillock has been split along its diameter by offsetting electrically the upper part of the picture from the lower part. The distance moved by the offset image was measured electrically.

allows us to measure the diameters of hillocks at frequent short time intervals (Fig. 3). To estimate the rate of secretion of individual glands we assume that each hillock is hemispherical, and calculate the volumes of the hemispheres from the measured diameters. Although this provides only an estimate of the volume of secretion, it emphasizes the large changes in volume that occur with small changes in the diameter of a hillock (volume of a hemisphere = $\frac{1}{12} \pi D^3$).

To examine the secretory response of the submucosal glands to nervous or pharmacological stimuli, we measure the increase of number of hillocks per cm^2 during baseline conditions and after a stimulus. This method allows us to measure the pattern of the secretory response of individual glands to different stimuli (Fig. 3). Our studies with the method show that an α -adrenergic agonist, phenylephrine, causes secretion from canine tracheal mucous glands. The effect is smaller than the stimulatory effect of electrical stimulation of cholinergic efferent fibers in the superior laryngeal nerve (11). The difference in the response to the two stimuli may be due to the fact that phenylephrine has a vasoconstrictive effect on the local blood supply to the trachea (Fig. 4).

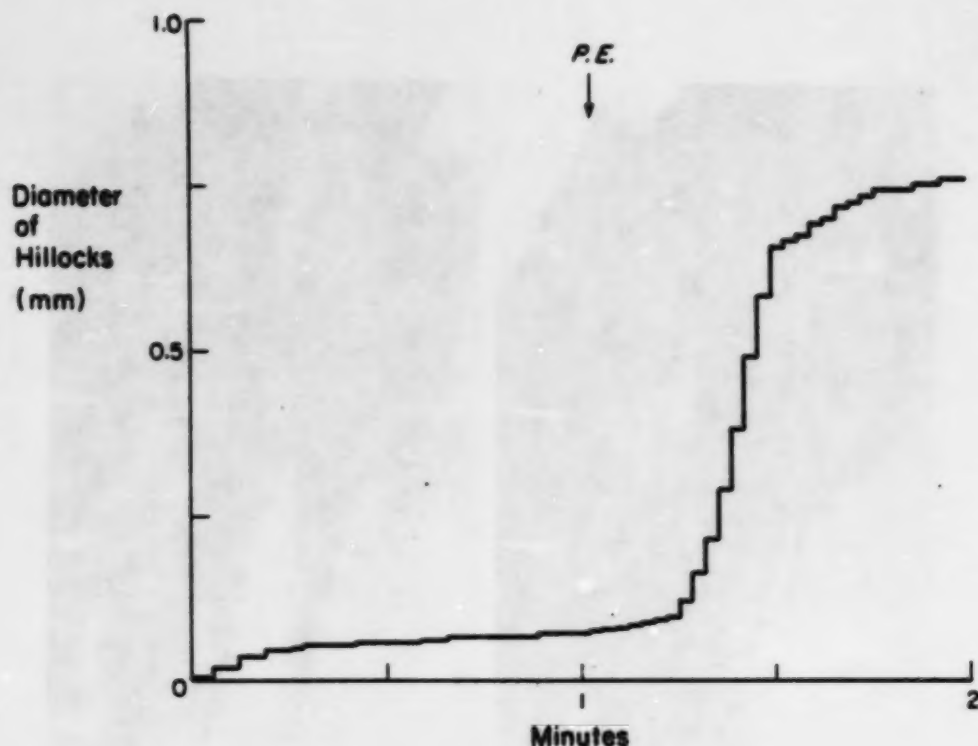


Figure 3. Graph of measurements of diameters of hillocks made over 2.5 seconds during a control period of 60 seconds and during a 60-second period after injection of 40 µg of phenylephrine (P.E.) into a cranial thyroid artery of the dog.

Another technique for studying submucosal gland secretion, which has been developed in our laboratory (31), allows the secretion from a single submucosal gland to be collected into a micropipette. Cat tracheal glands are studied because the secretions from these glands are watery and can be collected easily into a capillary tube. In anesthetized cats ventilated via the lower trachea, the tracheal mucosa is exposed and covered with HEPES oil equilibrated with water. To visualize the gland duct openings we stain them *in vivo* with neutral red dye (0.1% solution) applied to the luminal surface of the epithelium and observe them through a dissecting microscope. With experience we are able to recognize the gland duct openings by their elliptical shape and therefore we no longer use neutral red dye. To collect the secretion we use oil-filled constant bore pipettes bent at their ends to an angle of 30°-45°. The tips of the pipettes are fire polished to prevent damage to the epithelial surface. The tip of the pipette is placed over a gland duct opening, so that the opening is surrounded and can only discharge

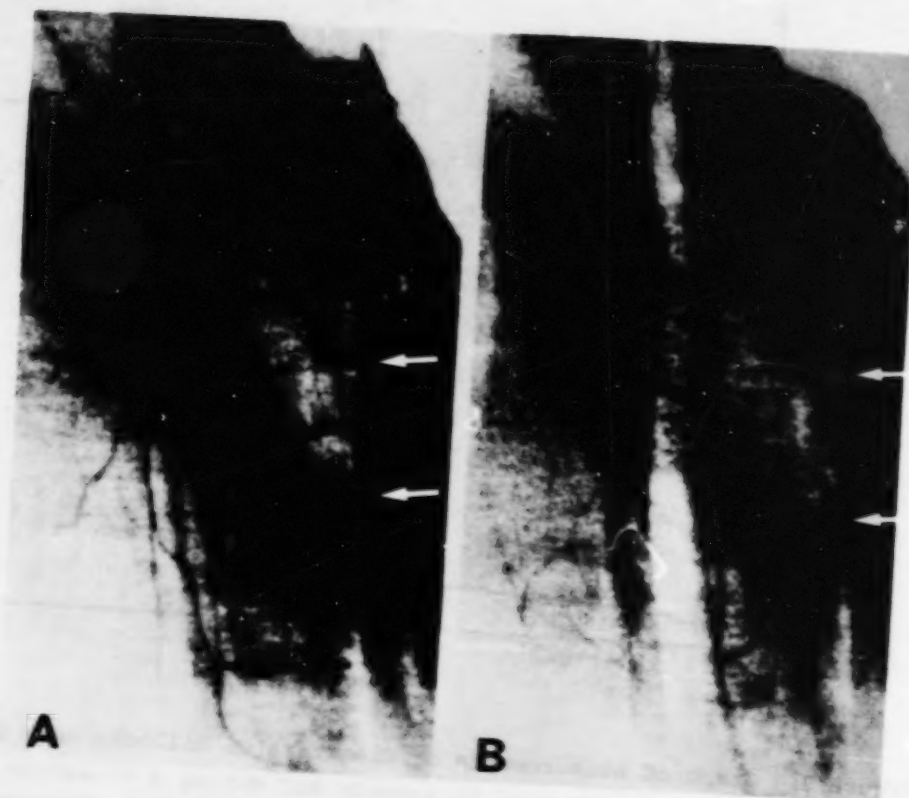


Figure 4. Angiograms of canine tracheal vessels produced by injecting dye through a cannula in the right cranial thyroid artery 1 minute after injection of 2 ml of saline (A) and 1 minute after injection of phenylephrine, 40 μ g in 2 ml of saline (B). Phenylephrine caused constriction of the tracheal vessels (arrows).

its secretion into the capillary lumen. We start the flow by applying slight negative pressure with a syringe to the end of the micropipette. Secretion is collected for 1 minute and then oil is aspirated into the capillary lumen to isolate the sample; two or three samples are collected into one micropipette and the volume of each sample is calculated by measuring the length of the fluid column using a Vernier micrometer and a stereomicroscope. The volumes of sequential 1-minute samples collected from the same gland duct vary very little over periods up to 4 hours. We used this method to show that the rate of secretion from the glands was increased by electrical stimulation of the vagus nerves and by intravenous injection of phenylephrine (30, 31).

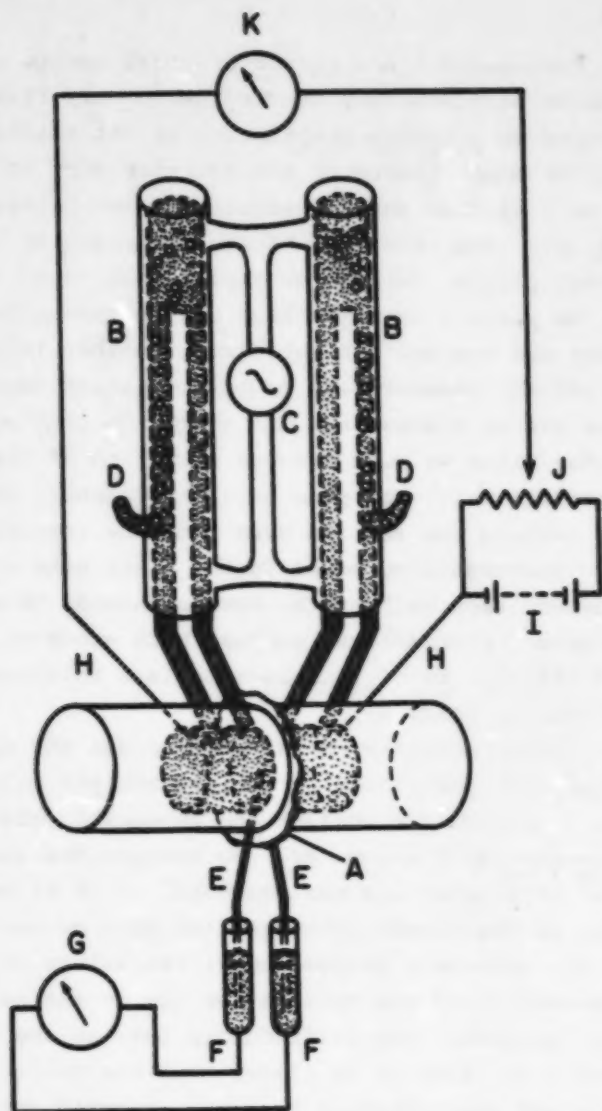


Figure 5. Using chamber, glass perfusion chamber, and electrical circuits. A piece (1.2 cm^2) of trachea (A) bathed on each surface by Krebs-Henseleit solution warmed to 37°C in a heating jacket (B) which is connected to a water pump and heater (C). The solution is circulated and oxygenated by bubbling it with 95% O_2 , 5% CO_2 (D). Electrical potential difference is measured with KCl-agar bridges (E) connected via calomel half cells (F) to an electrometer (G). The trachea is short-circuited by passing current to the electrolyte solution via saline-agar bridges (H) from an external source (I) regulated by an automatic voltage clamp (J) to keep the spontaneous electrical potential difference at zero. The short-circuit current is measured by a milliammeter (K).

IN VITRO TECHNIQUES

The effect of physiological and pharmacological agents on secretion of mucus cannot at present be adequately studied using only tissues in vivo. Therefore, we developed an in vitro preparation of cat trachea for the study of mucus secretion. We mount pieces of the anterior part of the trachea (exposed area, 1.3 cm^2) as flat sheets between the two halves of a modified Ussing chamber (Fig. 5). The pieces of tissue, composed of pseudostratified epithelium, submucosal glands, connective tissue, and cartilage, are approximately 1 mm thick. We place a thin parafilm ring between the epithelial surface of the tissue and one-half of the Ussing chamber to help form a seal between the tissue and the chamber, and to help minimize edge damage.

One-half of the Ussing chamber has six sharpened pins equally spaced around the medial edge which we push through a section of the trachea, pinning it to a block of paraffin wax. The pins hold the tracheal section flat across the chamber. After cutting the section free from the remaining trachea, we insert the pins into corresponding holes in the other half of the chamber to close it. We connect each half of the Ussing chamber to a glass perfusion chamber (MRA, Clearwater, Florida) and perfuse both sides of the chamber with 10 ml of oxygenated (95% O_2 , 5% CO_2) Krebs-Henseleit solution, warmed to 37°C to maintain viability (Fig. 5).

We measure electrical potential difference across the epithelium via two agar-KCl (1 M) bridges positioned in the electrolyte solution on either side of the trachea 2 mm from the surface and connected through calomel half cells to a high impedance millivoltmeter. We measure the short-circuit current (expressed as $\mu\text{A}/\text{cm}^2$ of tissue) via two agar-NaCl (0.15 M) bridges which carry direct current to the electrolyte solution from an automatic voltage clamp connected to the agar-salt bridges by silver/silver chloride junctions. To calculate the resistance of the tissues, we divide the potential difference by the short-circuit current. The relationship between the clamped voltage and the current needed to clamp it is linear over the range +40 mV to -60 mV. Potential difference and short-circuit current, measured every 15 minutes during the experiment, are used to monitor the viability of the tissue.

To study the effects of a drug on sulfated mucin secretion, and Cl^- and Na^+ fluxes, we measure sulfated mucin secretion, bidirectional fluxes of Cl^- , and bidirectional fluxes of Na^+ in separate pieces of tissue before and after the addition of the test drug.

$[^{35}\text{S}]$ sulfate is taken up by the mucus-secreting cells of the cat trachea and is released into the airway lumen bound to glycoproteins (15). For this reason, we use the secretion of bound ^{35}S into the luminal side of the chamber as a measure of sulfated glycoprotein secretion. We add 1.0 mCi sodium $[^{35}\text{S}]$ -sulfate to the submucosal side of the tissue; every 30 minutes we collect the

solution bathing the luminal side of the tissue and replace it with fresh Krebs-Henseleit solution. Samples are collected for a control period of 3-4 hours. The test drug is added to the submucosal side of the tissue for one 30-minute sampling period and subsequently washed out. Samples are collected for 1-2 hours after the drug is washed out. The samples obtained are placed in dialysis tubing bags (average pore radius permeability 24 Å, VWR Scientific) and dialyzed against distilled water to remove unbound [^{35}S]sulfate. Up to 30 of these samples are placed in 4 liters of distilled water; this water is exchanged six times during a 48-hour period. To each of the first four volumes of dialysis water we add nonradioactive sodium sulfate (0.5 g) to displace noncovalently bound [^{35}S]sulfate, and we add sodium azide (0.5 g) to prevent fungal and bacterial growth. The last two dialyses are against 20 mM phosphate buffer, which helps to disperse the mucins. On the completion of dialysis, a 0.6 ml aliquot of each sample is taken, mixed with 4.0 ml of scintillation fluid (PCS; Amersham) and the bound ^{35}S is counted in a β -scintillation counter (Liquid Scintillation System MK III, Searle Analytic). The output is expressed as counts per minute per square centimeter of tissue per hour ($\text{cpm}/\text{cm}^2\cdot\text{hr}$).

To measure the fluxes of Cl^- and Na^+ across the tissue, we add Krebs-Henseleit solution which contains either 5-10 μCi ^{36}Cl or 2-5 μCi ^{22}Na to one side of the chamber, and measure the rate of appearance of radioactivity on the other side. We obtain samples by collecting the solution from the appropriate side of the chamber and replacing it with fresh Krebs-Henseleit solution every 30 minutes. We collect samples for a control period of 2 hours, add the test drug to the submucosal side of the tissue for one 30-minute sampling period, and subsequently remove the drug by washing. Samples are collected for 1 hour after the drug is removed. A 0.6 ml aliquot of each of these samples is taken, mixed with 4.0 ml scintillation fluid, and the radioactivity counted in a β -scintillation counter. From the specific activities of the ^{36}Cl and ^{22}Na source solutions we are able to calculate the ion fluxes in micro-equivalents per square centimeter of tissue per hour ($\mu\text{Eq}/\text{cm}^2\cdot\text{hr}$). When we have measured the flux of an ion in one direction, we wash out both sides of the chamber and measure the flux of the same ion in the opposite direction, before and after the test drug, in the same piece of tissue.

All fluxes are measured under open-circuit conditions, except for a few seconds (less than 5) every 15 minutes when short-circuit current is measured. Electrical potential difference and short-circuit current are measured every 5 minutes during the 30-minute period that the test drug is in the chamber.

This new method for studying respiratory tract secretion has the following advantages over other in vitro methods: We can measure mucus output and ion transport in similar pieces of trachea from the cat, under well-controlled conditions for periods up to 10 hours. We can assess the viability of the

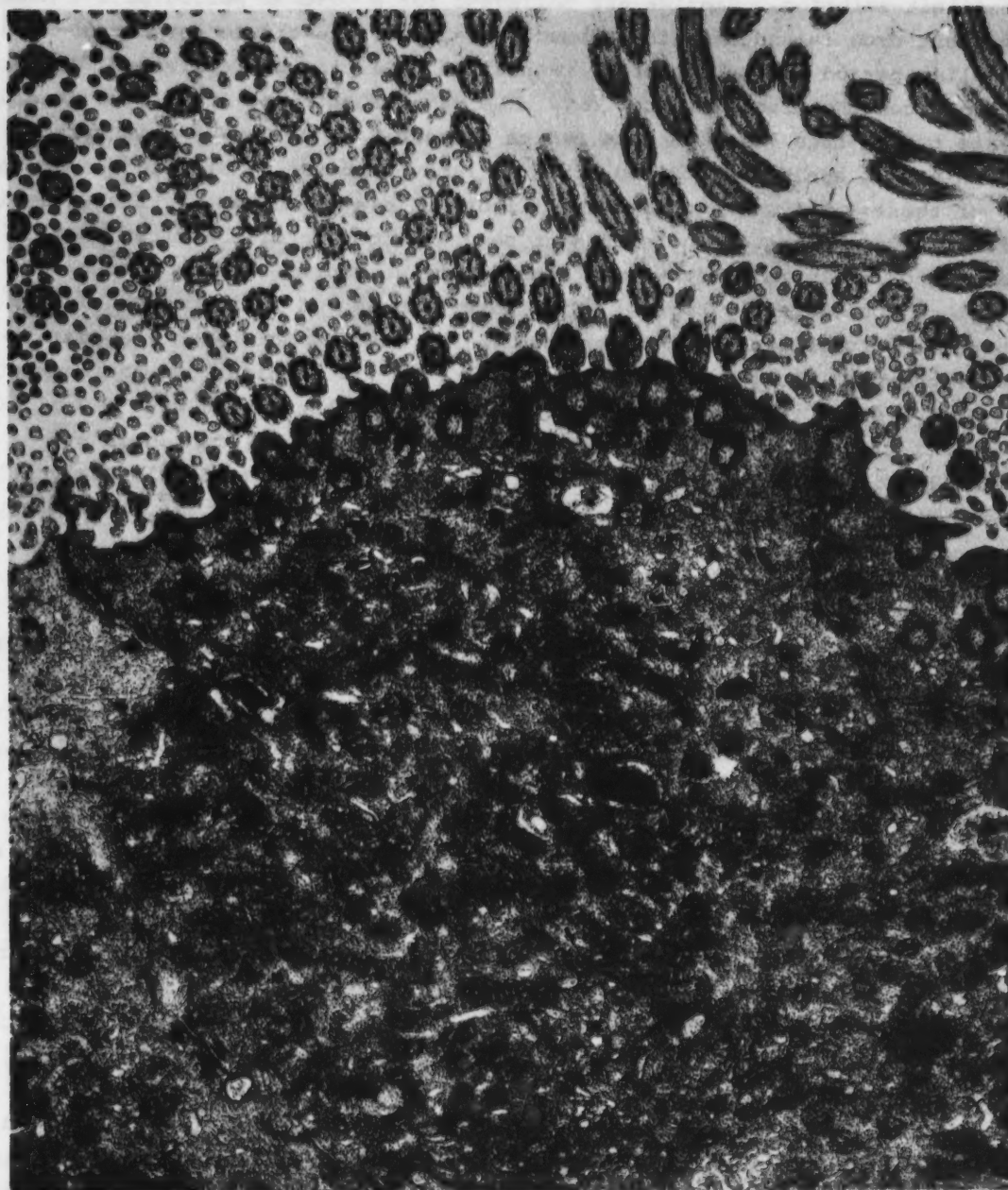


Figure 6. Electron micrograph of a ciliated cell prepared from canine tracheal epithelium which had been mounted in an Ussing chamber for 8 hours. The ultrastructure of the cell and junctions appear normal.

tissue by electrical measurements during the experiments and by electron microscopy after the experiments. Using this method we found that an α -adrenergic agonist (phenylephrine) and a β -adrenergic agonist (terbutaline) both stimulated the secretion of sulfated mucus and net ion movement into the lumen (9, 27). The effects of α -adrenergic and β -adrenergic stimuli were different. Phenylephrine caused large increases in the output of sulfated mucin and in the net fluxes of Cl^- and Na^+ toward the lumen; terbutaline caused a moderate increase in the output of sulfated mucin and a small increase in net Cl^- flux toward the lumen; it did not affect either unidirectional flux of Na^+ . From these findings we predict that phenylephrine will cause more ion-mediated water secretion than terbutaline. Thus, phenylephrine may act to produce more water in the respiratory tract secretions.

Originally we used trachea mounted in an Ussing chamber to study ion movement across canine tracheal epithelium (26) (Fig. 6). In epithelia, ion movement induces transepithelial movement of water by creating local osmotic gradients in the lateral intercellular spaces (13, 29). Therefore, we reasoned that active ion transport was likely to regulate the rate of water secretion into the airway. Changes in ion-mediated secretion of water may alter the consistency of respiratory tract fluid and change the depth of the periciliary fluid layer. The periciliary fluid layer is important for the regulation of mucociliary clearance. With too much periciliary fluid, the propulsive effect of the cilia will not reach the mucus gel layer; with too little periciliary fluid, the ciliary motion will be blocked by the weight and resistance of the gel layer. Thus, the regulation of water secretion into the periciliary fluid could be an important determinant of mucociliary clearance rates.

To study ion movement we used Ussing's short-circuit current method and the posterior membranous part of the trachea of dogs. We found net movement of Cl^- toward the lumen ($2.7 \pm 0.6 \mu\text{Eq}/\text{cm}^2 \cdot \text{hr}$) and net movement of Na^+ toward the submucosa ($0.8 \pm 0.2 \mu\text{Eq}/\text{cm}^2 \cdot \text{hr}$). These net ion fluxes measured under short-circuit conditions were associated with a spontaneous transepithelial electrical potential ($30.7 \pm 2.7 \text{ mV}$) and a short-circuit current ($108 \pm 8 \mu\text{A}/\text{cm}^2$) (26). Thus, Cl^- and Na^+ must be actively transported across canine tracheal epithelium. By forming local osmotic gradients ions may regulate movement of water into the tracheobronchial sections (5, 24).

Mediators and drugs which mimic the actions of the autonomic nervous system affect ion movement across the trachea. Histamine increased net movement of Cl^- and Na^+ toward the tracheal lumen; the response was prevented by an H_1 -antagonist but not by an H_2 -antagonist (20). Acetylcholine increased net movement of Cl^- and Na^+ toward the lumen; the response was prevented by small concentrations of atropine (19). Terbutaline, a specific β_2 -adrenergic agonist, increased net movement toward the lumen of Cl^- , but not of Na^+ ; the

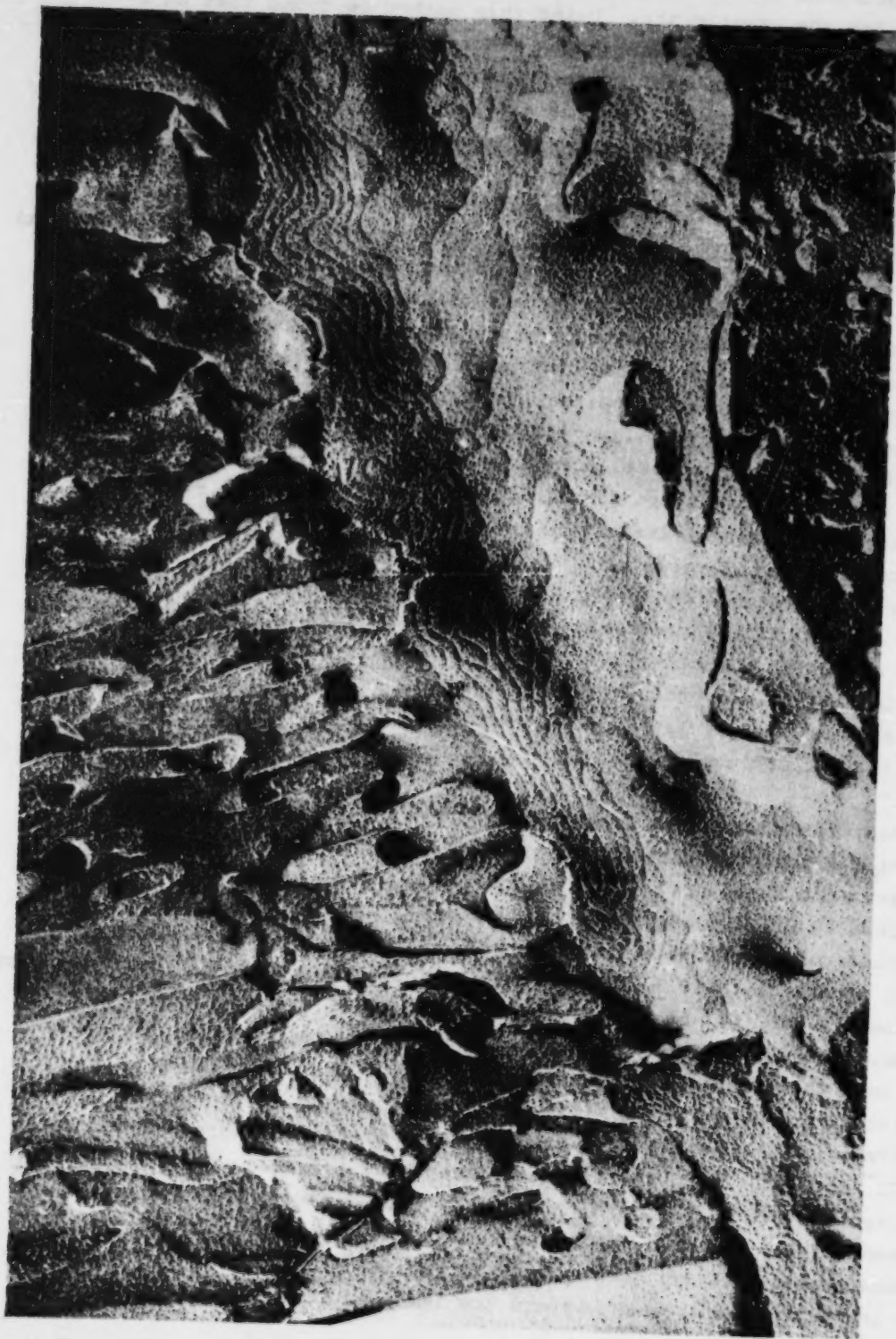


Figure 7. Freeze-fracture through the tight junction of a ciliated cell in cat trachea which was removed from an Ussing chamber after 6 hours. The junction is formed by parallel strands with a few interconnections.

effect was prevented by propranolol (6, 12). We speculate that ion-mediated water secretion into the airway can be altered by mediators and neurotransmitters.

Active transport in tracheal epithelium was affected by drugs which inhibit ion transport in other epithelia. Furosemide reduced net Cl^- movement toward the lumen when added to the submucosal side, but not when added to the luminal side of the trachea (8). Ouabain was bound to more sites on the submucosal membranes than the luminal membranes of the tracheal surface epithelial cells (32); the drug inhibits Na^+ transport when added to the submucosal side of the trachea but has little effect when added to the luminal surface of the epithelium. The transepithelial movement of Cl^- depends on co-existing active Na^+ transport, since net Cl^- movement is greatly reduced by replacement of Na^+ in the bathing solution (1, 21, 33) and by adding ouabain, an inhibitor of sodium pumps, to the submucosal bathing solution (1, 33). Further studies will be necessary to determine the cells responsible for the active ion transport and the exact systems involved.

We use the in vivo preparation of cat trachea in an Ussing chamber to study epithelial permeability and electrical properties and the pattern of tight junction strands in the same piece of epithelium (18). In each tissue we measure electrical potential difference, short-circuit current, and electrical resistance for periods up to 6 hours. Simultaneously, we measure the flux of ^{14}C -sucrose (an extracellular tracer) from submucosa to lumen during 30-minute periods. At a predetermined time during the experiment, we remove the tissue, fix it, and prepare it for freeze fracture. Small pieces of the trachea are fractured and replicated in a freeze-etch device (Balzers, Liechtenstein) with a double replica stage. We use electron micrographs of freeze-fractured tracheal tight junctions (Fig. 7) to quantitate the changes in tight junctions associated with changes in electrical resistance and permeability of the epithelium. We used this method to study the effect of removing Ca^{++} from the bathing solutions on the properties of the epithelium. The Ca^{++} -free solution caused a decrease in electrical resistance, an increase in permeability to ^{14}C -sucrose, and produced areas of disarray and reorientation of the tight junction strands (18).

SUMMARY

The control systems for the production and removal of respiratory tract secretions may be altered in disease. For instance, patients with cystic fibrosis do not adequately clear the sticky secretions which form in their airways, patients with severe asthma plug their small airways with altered mucus, and patients with chronic bronchitis produce excessive amounts of secretion that must be removed from the airway. Direct methods for studying

the regulation of the system in man are not available because the mucous glands are inaccessible and the secretions obtained at the mouth are contaminated. Previous methods for studying the respiratory tract secretions in animals did not identify the sources of the secretions. We have developed new techniques using animal tracheas in vitro and in vivo which allow us to study the submucosal glands. We have shown that the glands have a secretory response to cholinergic agonists and stimulation of the vagus nerve, and to α -adrenergic and β -adrenergic agonists.

We have shown that tracheal epithelium actively transports Cl^- and Na^+ and that the net movement of these ions toward the lumen is increased by autonomic agonists. Net movement of these ions may be an important control of ion-mediated water flux into the lumen. Furthermore, changes in the permeability of the tissue may modify ion-mediated water movement. We have shown that tight junctions can be reversibly altered by changes in their external milieu. We speculate that epithelial permeability may also be modified by the autonomic nervous system. In the future, we expect that a better understanding of the controls of airway epithelial functions will lead to advances in the treatment and prevention of airway disease.

ACKNOWLEDGMENTS

We thank our colleague Todd Lempert for preparing the electronmicrographs. The studies on tight junctions were performed in collaboration with Dr. D. S. Friend. Dr. Davis is the recipient of a NHLBI Young Investigator Research Award HL-21150. Some of the reported studies were supported by grants from the U.S. Public Health Service--Program Project HL-06285, Pulmonary SCOR Grant HL-19156, and HD-10445--and by Cystic Fibrosis Foundation Grants.

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DISCUSSION

DR. WASSERMAN: You've presented your mucus secretion in terms of ^{35}S label, although your autoradiographs would make you think that it was in mucus. How do you, in fact, know that your counts were in mucus?

DR. DAVIS: Gallagher et al. showed that ^{35}S is incorporated into mucus glycoproteins secreted into cat trachea in vivo. [Gallagher JT, Kent PW, Passatore M, Phipps RJ, Richardson PS: The composition of tracheal mucus and the nervous control of its secretion in the cat. Proc R Soc London (Biol) 192:49-76, 1975].

Mucus Glycoprotein Secretion by Tracheal Explants: Effects of Pollutants

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We have been using the tracheal explant system, developed several years ago in the laboratories of Dr. Lynn Reid (13) and others (2, 3, 5-7, 15), as a quantitative assay for monitoring effects of air pollutants and other pneumotoxins on the respiratory epithelium. We have used an "in vivo" approach, as indicated schematically in Figure 1, featuring exposure of animals (usually rats) in vivo to known amounts of pneumotoxin, followed by removal of their tracheae for assay of effects in vitro. With small animals such as rats, we routinely incubate the tracheae in tissue culture medium containing radioactively labeled precursors. The culture medium is then removed (Fig. 1) and an aliquot is treated with 5% trichloroacetic acid to precipitate the proteins present. With a suitable choice of labeled precursor and incubation time for the rat tracheal explants, essentially all of the acid-precipitable radioactivity in the medium is mucus glycoprotein, as we will demonstrate. What are the characteristics of such an assay? Figure 2 shows the time course of incorporation of [^{3} H]glucosamine by tracheal explants in such an assay system. The appearance of acid-precipitable glycoproteins in the culture medium can first be observed after about three hours. The incorporation rate is linear between about 8 hours of incubation and at least 50 hours, the maximum duration of incubation, as shown in Figure 2. In some experiments the time course has remained linear for as long as 96 hours (data not shown). For routine assay purposes we generally incubate tracheal explants for about 24 hours. This is a convenient duration of in-

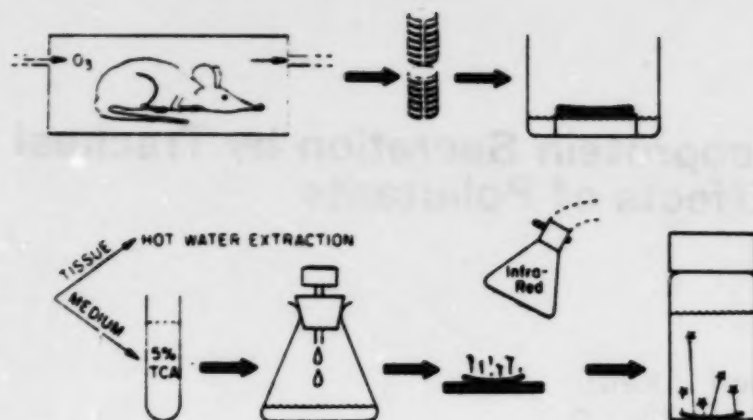


Figure 1. Schematic diagram of a typical "in vivo" experiment. Rats are exposed to a pneumotoxin (in this case, ozone) *in vivo*. Tracheae are then excised and their secretion rates are determined *in vitro* in tissue culture medium containing suitable labeled precursor(s). After incubation, an aliquot of medium is precipitated with trichloroacetic acid and filtered. The filters are dried and counted by liquid scintillation (cf., ref. 12).

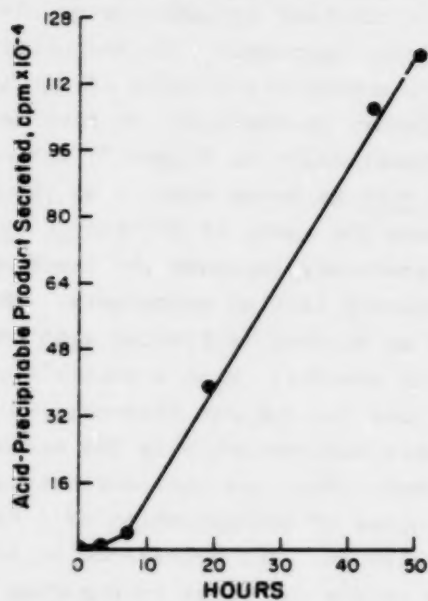


Figure 2. Time course of incorporation of [^3H]glucosamine by rat tracheal explants into acid-precipitable glycoproteins released into the culture medium (cf., ref. 12).

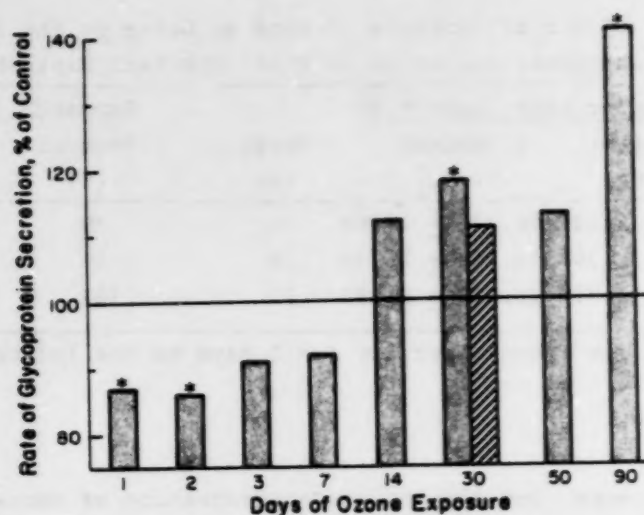


Figure 3. Glycoprotein secretion rates (expressed as percent of control value = 100%) of tracheal explants prepared from intact rats exposed to 0.8 ppm of ozone for the lengths of time indicated on the abscissa (cf., ref. 12). The hatched bar at 30 days represents results from a separate experiment to indicate the reproducibility of the results obtained by this technique.

cubation in that enough radioactivity is incorporated to enable accurate quantitation, and the incorporation is clearly first order with respect to time, so that an actual rate of incorporation is being measured.

How can we apply this system to air pollution toxicology? Figure 3 illustrates the results of an experiment in which rats were exposed to 0.8 parts per million of ozone continuously for periods ranging from 1 to 90 days of exposure, and their response was evaluated by the tracheal explant assay. There is an initial decrease in the rate of glycoprotein secretion by tracheal slices as compared to the rate of secretion by slices prepared from matched control rats. This decrease lasts for 2 to 3 days, as evaluated by statistical significance; the secretion rate once again returns to the control values after a period of about 5 to 10 days (in different experiments). If the rats are exposed to ozone for periods longer than about a week, the secretion rate of their tracheal slices becomes greater than that of the controls. The secretion rate seems to increase continuously with longer durations of exposure, up to 90 days in Figure 3, and up to 180 days of exposure in other experiments we have performed. The hatched bar at 30 days shows the results of a separate exposure performed some 6 months after the one that had given rise to the rest of the data presented in Figure 3.

The tracheal explant system is sensitive not only to the duration of

Table 1. Effect of Exposure of Rats to Ozone on the Rate of Glycoprotein Secretion by Their Tracheal Explants

Ozone concentration (ppm) ^a	Secretion rate, mean $\pm$ SD		Total (n)	Exposed/control (%)	P value
	Control (cpm)	Exposed (cpm)			
0.6	19,110 $\pm$ 2,230	16,972 $\pm$ 2,269	24	89	<0.02
0.4	14,913 $\pm$ 2,309	14,327 $\pm$ 2,118	24	96	NS ^b
0.2	16,870 $\pm$ 2,770	17,034 $\pm$ 2,503	22	101	NS

^aExposures were for 8 hours per day for 3 days to the indicated concentration of ozone.

^bNot significant.

exposure of rats to ozone, but also to the concentration of ozone to which the rats are exposed. The dose-response characteristics of this system to ozone after 3 days are shown in Table 1. A statistically significant decrease in secretion rate is observed with 0.8 parts per million of ozone (see Figure 3) and with 0.6 parts per million of ozone (Table 1). An insignificant decrease is observed at 0.4 parts per million and no effect is seen at 0.2 parts per million (Table 1). The data of Table 1 also illustrate one of the difficulties in working with the tracheal explant system. Comparisons between matched controls and experimental rats are very precise within a given experiment. In a single experiment the size of tissue slices and the matching of rats can be controlled rigorously. A single batch of medium can be used so there are minimal errors introduced by pipetting and addition of labeled precursor to the medium. On the other hand, comparisons between separate experiments are much more difficult, as shown in Table 1. Small differences in technique from day to day result in variations in the absolute value for control rats such that data within a chronic exposure regimen must usually be normalized to control values; hence, the expression of the data in Figure 3 as percent of control. In addition, growth of the rats within a given chronic exposure experiment is appreciable and can also affect these results.

The tracheal explant system can be used with animals other than rats. We have demonstrated elsewhere (10) that tracheal explants from a wide variety of experimental animals, as well as human tracheal and/or bronchial explants, can be used in this system. In Table 2 we present a comparison of tracheal explant secretion rates for multiple tracheal slices prepared from individual bonnet monkeys exposed to either 0, 0.5, or 0.8 parts per million of ozone for 7 days. While the increased secretion rate after 7 days suggests that

Table 2. Tracheal Explant Secretion Rates for Tracheal Slices from Monkeys Exposed to Ozone

	Controls	0.5 ppm O ₃	0.8 ppm O ₃
Secreted precipitable labeled molecules after 7 days of O ₃ exposure	12,362 ± 2,108	16,679 ± 3,767	21,949 ± 6,383

there may be a species difference between monkeys and rats, we obviously need to look at many more monkeys to confirm these results. Nonetheless, clearly the technique is applicable to animals other than rats, especially non-human primates.

We can also use this system to study effects of pollutants other than ozone. For example, we have studied effects of exposure of rats to sulfuric acid aerosols, alone and in combination with ozone (11). Some representative results we have observed in this assay with sulfuric acid aerosols and/or ozone are presented in Table 3. While 0.5 ppm of ozone by itself causes a decrease in the observed secretion rate after three days, the combination of sulfuric acid aerosol with ozone causes a significant increase in this measured value. Similarly, these agents potentiate one another after 14 days of continuous exposure (Table 3). Sulfuric acid aerosol, by itself, is without affect on matched rats, as measured by their tracheal explant secretion rate, at these levels tested (11). Thus, exposure of rats to the combination of ozone and sulfuric acid aerosol (0.5 ppm of ozone; 0.01-5 mg/m³ of sulfuric acid aerosol, ref. 11) causes changes in the tracheal explant secretion rate that are not only quantitative, but are qualitative over the 2- to 3-day exposure interval. We are presently vigorously studying the biochemical and/or cellular basis of such pollutant-induced changes in secretion rates.

Once we had documented quantitative changes in mucus secretion rates by tracheal explants from rats exposed to various pollutants, we turned our attention to the question of whether there was also a qualitative change in the mixture of glycoproteins being secreted by the explants. To separate the mucus glycoproteins into various fractions on ion-exchange columns, it was first necessary to solubilize these compounds. In the absence of prior treatment to solubilize the glycoproteins, yields from ion-exchange columns were very low and erratic. Other workers have used reduction and alkylation of sulfhydryl groups in the protein backbone involved in glycoprotein cross-linking for this purpose (1, 14); however, since we were most interested in

Table 3. Rate of Secretion of Mucus Glycoproteins by Tracheae from Rats Exposed to H₂SO₄-O₃ Mixtures

Days exposed <u>in vivo</u>	H ₂ SO ₄ (mg/m ³)	O ₃ (ppm)	Rate of secretion (% of control value) ^a	P value
3	1.1	0.5	127	<0.005
14	1.1	0.5	132	<0.001
3	1.1	None	100	NS ^b
3	None	0.5	84	NS
14	None	0.8	112	NS

^aTracheal explants were incubated 22-24 hours; in all cases, n = 14 slices.

^bNS, not significant.

determining changes in the carbohydrate side chains rather than the protein backbone, we chose limited digestion with papain as our initial solubilization step, as shown schematically in Figure 4. After papain digestion we can chromatograph the resultant mixture of glycoproteins on DEAE-cellulose and recover the applied radioactivity essentially quantitatively. As shown in Figure 5, an idealized version of such a column, the [³H]glucosamine-labeled glycoproteins separate into one neutral and two acidic glycoprotein fractions under the conditions we initially used for this purpose (a linear gradient from 0 to 1 molar lithium chloride in 50 mM sodium acetate-acetic acid buffer at pH 4.4). We observe three peaks (A, B, and C) that are eluted at the same positions from the DEAE-cellulose column whether we use [³H]glucosamine or [¹⁴C]threonine as the precursor for the glycoproteins. When [³⁵S]sulfate is used as the labeled precursor, most, if not all, of the label appears in the most acidic fraction from the DEAE-cellulose column, the fraction that we call peak C. This material is chromatographically indistinguishable from peak C labeled with [³H]glucosamine or with [¹⁴C]threonine. Both of these two acidic peaks are eluted from the column at positions distinctly different from those of hyaluronic acid or chondroitin sulfate treated in an identical manner, added as internal standards and assayed chemically by determination of their constituent uronic acid components. If a mixture of the two acidic fractions is incubated at 37° in an appropriate buffer overnight for 18-24 hours, then rechromatographed, their elution positions are unchanged from the original chromatographic mobilities observed. If the same mixture of fractions B and C is incubated in buffer containing neuraminidase from *Vibrio cholerae*, an enzyme that specifically cleaves sialic acid residues from the non-reducing end of oligosaccharide chains, we then observe only one peak after incubation; this single peak is chromatographically indistin-

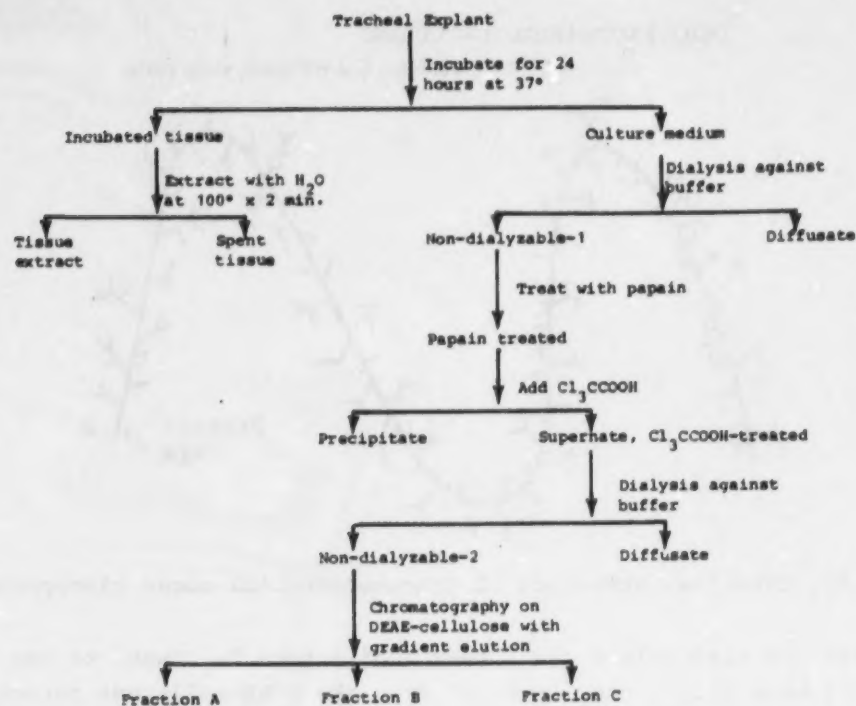


Figure 4. Diagrammatic representation of the techniques used to purify mucus glycoproteins from the culture medium of tracheal explants.

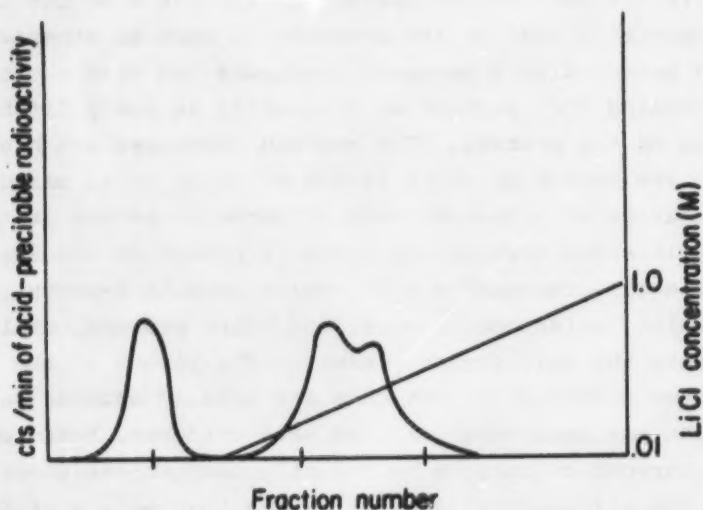


Figure 5. Idealized representation of the elution of labeled glycoproteins, treated as in Figure 4, from a column of DEAE-cellulose.

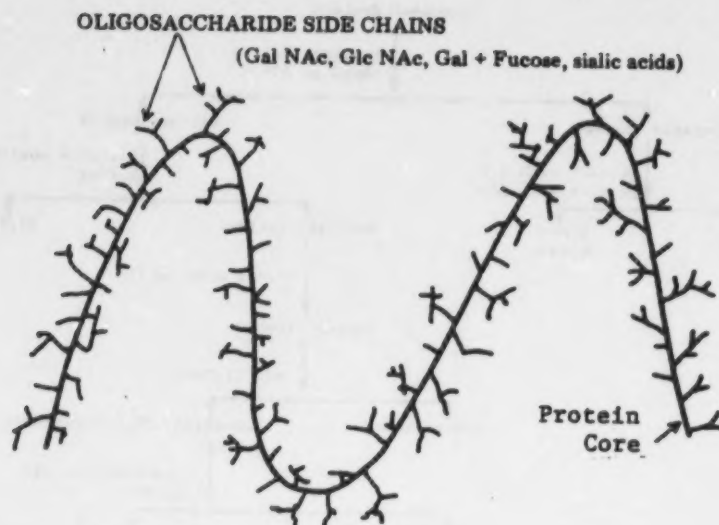


Figure 6. Idealized structure of tracheobronchial mucus glycoproteins.

guishable from the less acidic peak, peak B of Figure 5. Thus, we can conclude that the more acidic peak (peak C) from the DEAE-cellulose column contains glycoproteins containing oligosaccharide side chains with terminal sialic acid residues in their structure. The sulfur-35 labeling experiment suggests that, in addition to its sialic acid content, the more acidic fraction is also more heavily sulfated than is fraction B.

In Figure 6 we indicate schematically the structure of the tracheobronchial mucus glycoproteins that we are studying in such an experiment. They are long extended proteins in a backbone configuration with short oligosaccharide side chains coming off, perhaps as frequently as every fifth or sixth amino acid residue of the protein. The protein backbones are high in serine and threonine, to the extent of about 40-50% of their total amino acid content, and the oligosaccharide side chains occur attached to serine or threonine residues in ester linkages through the hydroxyl groups of the serine or threonine residues. The oligosaccharide side chains contain N-acetylglucosamine, N-acetylgalactosamine, galactose, fucose, and, when present, sialic acids. These are presumably the only sugars present. The length of the side chains is a subject of some controversy, but they are usually assumed to be approximately six or eight residues long (8). We have evidence, based on cleavage of the serine or threonine linkages to the oligosaccharides under alkaline conditions, that the oligosaccharide side chains that we are studying are relatively homogeneous in size, as indicated by their mobility on columns of Sephadex G-50 (data not shown).

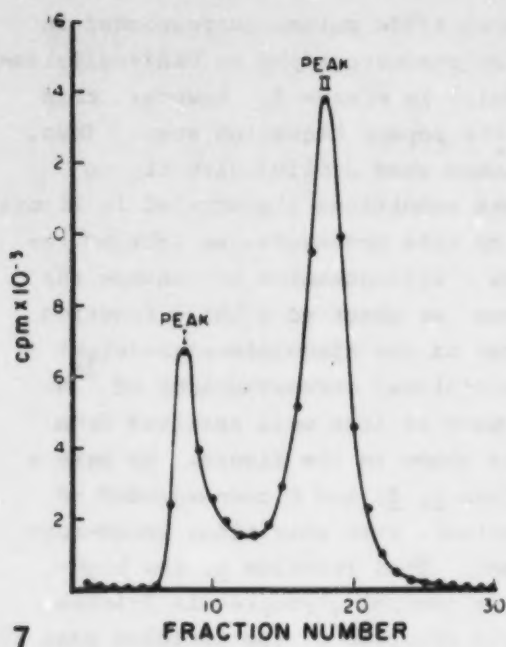


Figure 7. Elution of [³H]glucosamine-labeled glycoproteins, prepared from exhaustively dialyzed tracheal culture medium, from a column of BioGel A150m (cf. ref. 12).

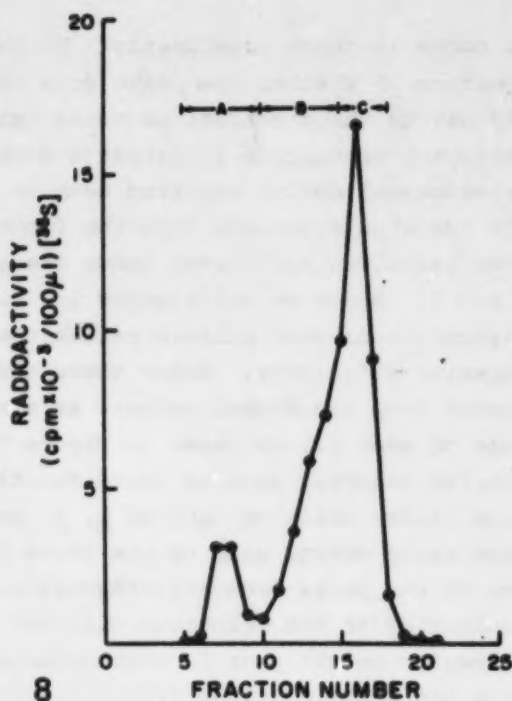


Figure 8. Elution of ³⁵S-labeled glycoproteins, prepared as in Figure 4, from a BioGel A150m column.

One technique that has been used extensively for the characterization of mucus glycoproteins secreted by cultured tracheal explants has been fractionation upon sizing columns, usually either agarose or polyacrylamide (4-6, 9). Under appropriate conditions of chromatography, two peaks of mucus glycoproteins are observed to be eluted from such sizing columns. The first peak, which elutes at approximately the void volume of the column, is extremely large and presumably represents aggregated glycoproteins. The second peak, which is included in the column, may also be shown to be a glycoprotein fraction (6). The ratio of glycoproteins in the two peaks has been used by others (5), as well as by ourselves (12), as a probe for observing changes in the structure of glycoproteins being secreted by tracheal explants. We have published (12) evidence that chronic exposure of rats to ozone causes changes in the ratio of the two peaks from a BioGel A150m column such that the lower-molecular-weight included peak is augmented at the expense of the first peak, as shown in Figure 7. In simpler terms, there was a shift in the relative number of counts from peak I to peak II upon exposure of the rats

to ozone in those experiments. We therefore turned our attention to the question of whether the peaks from the BioGel A150m column corresponded in any way to the fractions we could isolate by chromatography on DEAE-cellulose using the techniques illustrated schematically in Figure 4. However, this experimental design required that we omit the papain digestion step. Thus, the isolated fractions from the BioGel columns were applied directly to DEAE-cellulose and eluted under the gradient conditions illustrated in Figures 4 and 5. Since we anticipated low yields by this procedure, we labeled the glycoproteins with sulfate rather than with [³H]glucosamine to enhance the counting efficiency. Under these conditions, we observed a third fraction eluted from the BioGel column, as a shoulder on the high-molecular-weight side of peak II, as shown in Figure 8. Thus BioGel chromatography of ³⁵S-labeled material gave us three fractions, more or less well resolved from each other, which we labeled A, B, and C as shown in the figure. To make a long story short, each of the three fractions A, B, and C corresponded to one of the peaks from the DEAE-cellulose column, with only minor cross-contamination of the fractions with one another. Thus fraction A, the high-molecular-weight peak I, corresponded to the neutral glycoprotein fraction from the DEAE-cellulose fraction. Similarly fraction C, the included peak II, corresponded to the first acidic glycoprotein fraction B from DEAE-cellulose. Finally fraction B, the high-molecular-weight shoulder on peak II, corresponded to fraction C, the most acidic glycoprotein fraction from DEAE-cellulose. Thus, we can express the results of our earlier experiments, in which we had shown a shift in the ratio of the glycoproteins from peak I to peak II after exposure of rats to ozone, in more biochemical terms. The chronic exposure of rats to ozone apparently induces a shift in the composition of the mucus glycoproteins being secreted by their tracheal explants such that the product is relatively more acidic than is the mixture of mucus glycoproteins from control rat explants. This result is not unexpected (11, 13), considering the results of others who have exposed rats to various irritant stimulants under chronic conditions.

We have recently improved our DEAE-cellulose chromatographic techniques by changing to a less steep lithium chloride gradient. Under these conditions one neutral fraction is still eluted, but four acidic fractions are now eluted rather than two. Each of these four acidic fractions is different from the other as evaluated by cellulose acetate electrophoresis or by re-chromatography on DEAE-cellulose. We are currently using immobilized lectin columns as a technique for probing the structure of the sugars on the oligosaccharide side chains within the components of the various fractions. We have been able to document shifts in the ratios of these chromatographic peaks relative to one another upon exposure to ozone in this new system. For example,

as shown earlier (cf. Figure 3, Tables 1 and 3), short-term acute exposure to ozone at 0.5 parts per million for three days results in a significant decrease in the mucus secretion rate by the tracheal slices. When the papain-treated glycoproteins from control and exposed rat tracheal explants are chromatographed on DEAE-cellulose, we find a shift to a mixture that is relatively deficient in acidic mucus glycoproteins from the tracheae of ozone-exposed rats. Experiments are currently in progress to try to elucidate which acidic fraction is being decreased under these circumstances; preliminary data implicate the sialic acid-containing fraction as being present in relatively smaller amounts.

In conclusion, we have developed the tracheal explant system as a technique for quantitating the effects of exposure to air pollutants and other pneumotoxins on the respiratory airways of small laboratory animals, and on non-human primates. These techniques are relatively easy to perform and give reproducible quantitative results. Thus, any airborne noxious agent can be evaluated with this system. We are currently concentrating our attention on trying to elucidate the molecular basis for changes in secretion rates both in terms of decreased secretion rates in short-term acute exposures to ozone and increased secretion rates in longer-term chronic exposure regimens to various pollutants. These techniques promise to have a great deal of value both for mission-oriented research (screening of airborne pollutants, gasses, and chemicals for effects of the respiratory airways) and for basic research studies (elucidation of the structure of tracheobronchial mucus in health and disease).

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DISCUSSION

PARTICIPANT: Why did you seem to use electrophoresis more?

DR. LAST: If we take the materials before we digest with papain, they don't permeate into SDS or conventional polyacrylamide gels in our hands. After papain digestion, we found that the convenience and ease of electrophoresis more than made up for the problems of quantitation where you can't recover all the materials. For routine work, we used cellulose acetate for screening purposes. However, for most of our studies we are trying to quantitate glycoproteins made; we're much more comfortable with the ion-exchange columns, where we can get quantitative recoveries. The literature on how to do the gel electrophoresis for mucus glycoproteins is in agreement with most of our experience, and that is that the yields are low--low and not reproducible.

DR. HU: The first question is about the trachea rings. Do you see any difference if you pick up the first ring or the last rings from the trachea?

DR. LAST: We're not using rings, we're using slices. For our normal size rat, we get either two or three slices. The comparison is: Is there a difference between the laryngeal and the carinal end of the trachea? In our hands, no.

DR. HU: There's no difference?

DR. LAST: Not in terms of the parameters that we're measuring. I don't know how much of what we look at comes from the epithelial layer or how much comes from glands. That, certainly, is a germane question, especially if the labeled glycoproteins were coming from glands. But that kind of observation leads me to believe that we are not dealing with a lot of glycoprotein coming from the glands. I can go further than that. If we used bronchi from larger animals, if we compared tracheae and bronchi, for example, from a monkey, we did not see any difference, if we kept the slices identical in size.

DR. HU: What is the size of your column?

DR. LAST: Very small, between 1.5 and 3 cm of depth and about 1 cm in diameter with the DEAE-cellulose. These are all very much micro techniques.

DR. HU: What was the flow rate?

DR. LAST: About 1-1.5 milliliters per minute. The rate of flow affects the resolution slightly.

DR. NETTESHEIM: I didn't quite understand how you exclude in these in vitro studies the synthetic contribution of the connective tissue and the cartilage, which are quite active and produce a lot of material that goes into the culture. Have you tried, for example, to strip the epithelium and see what the contribution is of the stripped piece?

DR. LAST: We excluded proteoglycan synthesis because of the animal we chose and the time of incubation in culture. Gallagher and Phipps' work, for example, in rabbits suggests that you would be seeing a good deal of proteoglycans if we incubated for longer times. Chemically, we can rule out more than 5% proteoglycans in our system. We've looked for it and it's not there. As I've said, the yields here are essentially quantitative, so we're not losing any. When we did comparative studies with other animals, we did see more acidic components coming off the DEAE-cellulose column, which I presume may be from proteoglycans, but in the worst case it's no more than 20% of the total if incubation is for about 24 hours. The yield of presumptive glycosaminoglycans increases if we incubated for 72 or 96 hours. We have chosen our conditions, either luckily or fortuitously, so that proteoglycan synthesis by the cultured tracheae is not a problem.

Mucus and Surfactant Synthesis and Secretion by Cultured Hamster Respiratory Cells

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Establishment of eukaryotic cells in monolayer culture as experimental models has contributed significantly to our current understanding of growth control and regulation of specialized functions in mammalian cells. The possibility that single cell types can be examined free from the heterogeneity of host tissue and yet retain their biologic function is a major advantage of tissue culture techniques. Unfortunately, the mammalian lung is a complex structure with different cells possessing numerous physiologic roles (3) and therefore provides a difficult primary source of tissue from which to selectively dissociate particular cell types. Of special interest are two classes of respiratory epithelial cells whose key functions are the synthesis and secretion of mucus glycoprotein or pulmonary surfactant, each essential to normal lung maintenance. Mucus is a viscous material which protects the tracheobronchial epithelium from inhaled particles and pathogenic microorganisms by trapping them and permitting their removal via ciliary activity. Surfactant or disaturated (primarily dipalmitoyl) phosphatidylcholine is released onto alveolar surfaces apparently by type II alveolar cells to reduce the surface tension at the air-liquid interface, preventing alveolar collapse. Mucus-secreting cells have until recently (6) not been isolated and grown in pure culture, restricting basic studies on the biology of the cell as well as properties of mucus production under normal and abnormal conditions. Attempts at cultivating type II alveolar cells, the major in vivo contributor of surfactant, have met with limited success (4, 7, 10).

Type II cells in culture lose their specialized function and characteristic multilamellar bodies during early passage, and these cultures are often contaminated with fibroblasts. Transformed cells which apparently arise during passage of the type II cell cultures have been investigated (12), but these cells are clearly different from the original parent cell type. In this paper we discuss selected properties of the mucus-secreting cell which we have isolated and passaged in culture for the first time (6) and report on the application of similar methodology to derive from hamster lung tissue proliferating cells which maintain a specialized capacity to synthesize and secrete surfactant.

MATERIALS AND METHODS

Respiratory Cell Cultures

Hamster tracheal cells were isolated by the dissociated cell rescue technique previously described (6) and maintained as monolayer cultures in nutrient mixture F-12 (GIBCO, Grand Island, N.Y.) with 10% fetal calf serum (FCS; GIBCO) plus penicillin G (100 units/ml) and streptomycin sulfate (100 µg/ml). This isolation procedure requires repeated protease treatment of tracheal rings and collection of dissociated cells by sequential gradient centrifugation.

Primary cultures of hamster lung epithelial cells were prepared by a similar isolation procedure using tissue from a newborn litter of Syrian Golden hamsters (Engle Laboratory Animals, Farmerburg, Ind.) less than one day of age. Lungs were pooled, rinsed several times in nutrient mixture F-12 containing 10% FCS and antibiotics, and minced into smaller fragments prior to repeated protease treatment and gradient centrifugation. Dissociated cells were suspended in F-12 medium containing 10% FCS and supplemented with vitamin A (2 µg/ml; Sigma Chemical Co., St. Louis, Mo.) and epidermal growth factor (10 ng/ml; Collaborative Research Inc., Waltham, Mass.) during initial growth. These lung-originated cells as well as the initial tracheal cells were seeded in appropriate tissue culture vessels and grown to confluence with half-medium changes at two-day intervals. The latter procedure, which provided cells with half-conditioned and half-fresh medium during the earliest stages of growth, was beneficial for monolayer formation. Cloning of lung cells was accomplished by releasing cells from the vessel surface during primary culture with 0.02% ethylenediaminetetraacetate (EDTA) in phosphate buffered saline (PBS, pH 7.8) and plating them at low density in 100 mm petri dishes. Individual penicylinders were placed over isolated cell growth and sealed with silicone grease. Cells were washed in PBS and treated with EDTA-

PBS prior to transfer to culture vessels containing fresh growth medium. All subsequent growth and passage of cells occurred in nutrient mixture F-12 containing 15% FCS plus antibiotics. These lung epithelial cell cultures were maintained for five to seven passages after which cells increased in size, became more granular, and failed to divide. On one occasion, a transformed cell clone spontaneously emerged during this senescence of the epithelial cultures. Established lines of lung cells from rat (L2), cat (AKD), and human (A549) reported to be of type II alveolar cell origin were obtained from the American Type Culture Collection, Rockville, Md. These cells were routinely grown in F-12 containing 10% FCS with antibiotics. Generally, all cultures were kept at 37°C in an atmosphere of 5% CO₂ in air.

Other Cell Cultures

Primary cultures of chick embryo cells were prepared in Minimum Essential Medium (MEM; GIBCO) plus 10% FCS as previously described (1) and used during the third passage. Baby hamster kidney (BHK) and mouse (L25) established cell lines were also maintained in this medium. These cells were examined for their ability to synthesize phosphatidylcholine and derivatives.

Radioisotopes

N-acetyl-D-galactosamine [acetyl-³H, 1.5 Ci/mmol] and L-serine [U-¹⁴C, 156 mCi/mmol] were purchased from ICN Radioisotope Division, Irvine, Calif. Choline chloride [methyl-³H, 69.5 Ci/mmol] was supplied by New England Nuclear, Boston, Mass.

Detection of Glycoprotein Secretion

Hamster tracheal cells suspended in MEM with 10% FCS were seeded in MultiWell (Falcon, Oxnard, Calif.) plates (0.5 ml volume/well, 150,000 cells/ml), and monolayer cultures were later radiolabeled with [³H]N-acetyl-D-galactosamine (2.4 µCi/well) and [¹⁴C]L-serine (0.8 µCi/well) for 24 hours. The culture fluid was removed from each well, clarified by centrifugation, and precipitated with 10% trichloroacetic acid prior to sodium dodecyl sulfate-polyacrylamide gel electrophoresis as previously detailed (6). Gels were sliced into 1 mm fractions and radioactivity determined by liquid scintillation spectrometry.

Phospholipid Bioassay

Synthesis of lecithin (phospholipid) and its derivatives was measured by incorporation of radioactive choline into phosphatidylcholine. For this

biosynthetic determination, all cell types, including lung cell cultures normally passaged in F-12 medium, were grown to confluence in MEM containing 10% FCS. Cell monolayers were washed once with serum-free MEM and incubated under the latter conditions for 20 hours with 1.0 μCi [^3H]choline per 75 cm^2 flask. Monolayers were then washed twice with PBS, gently trypsinized, and total cell numbers determined by counting with a hemacytometer. Cells were centrifuged at 400 x g for 10 minutes and each pellet transferred to a Potter-Elvehjem tube and homogenized for 10 minutes at room temperature in a 2:1 chloroform-methanol mixture (v/v) according to the Folch method (5). The extract was mixed with one-fifth its volume of 0.05% CaCl_2 and allowed to separate into two phases at 4°C overnight. The upper phase and interphase were discarded. The lower phase was carefully washed three times with upper phase solvent. The lipid sample was evaporated to dryness under a stream of nitrogen and redissolved in 2:1 chloroform-methanol mixture (v/v). All samples were stored at -20°C.

To compare total phospholipid synthesis with secretion among the various lung cell cultures, each cell type was seeded in 50 cm^2 petri dishes and grown as described above. Twenty-four hours after addition of radioactive choline, cells were separated from the culture fluid and lipids extracted from the cell fraction by the Folch procedure. The fluid containing secreted phospholipid was mixed with one volume of methanol and two volumes of chloroform, shaken well, and placed at -20°C overnight. Lipids in the lower phase were isolated as previously indicated.

Phospholipid Separation by Thin-Layer Chromatography

Lecithin was identified by one-dimensional thin-layer chromatography (TLC). Radioactive samples were applied to a 20 x 20 cm precoated silica gel 60 TLC plate (0.25 mm thickness). Dipalmitoyl phosphatidylcholine (Sigma Chemical Co., St. Louis, Mo.) and a mixture of lecithin and sphingomyelin (Sigma) were routinely included as standards. Plates were developed for 90 minutes with a 65:25:4 chloroform-methanol-water mixture (v/v) and dried for 30 minutes at room temperature. Spots were stained by 2,7-dichloro-fluorescein (Sigma) dissolved in acetone and detected by ultraviolet light illumination. The spot correlating with the R_f value of lecithin was scraped into ACS scintillation fluid (Amersham, Arlington Heights, Ill.) for radioactive determination.

Isolation of Disaturated Phosphatidylcholine

Since lecithin and its derivatives have similar R_f values and are difficult to separate by TLC, the method of Mason and co-workers (8) was employed to isolate disaturated phosphatidylcholine (DPC). In general, the lipid

sample was reacted with 3.1 mg osmium tetroxide (Sigma) in 0.5 ml of carbon tetrachloride for 15 minutes at room temperature. This material was evaporated to dryness under a stream of nitrogen and redissolved in 0.5 ml of 20:1 chloroform-methanol (v/v). Individual samples were then placed on an aluminum oxide (100-200 mesh, Bio-Rad Laboratories, Richmond, Calif.) column and neutral lipids were eluted from the column with 20:1 chloroform-methanol (v/v). DPC was then released with 70:30:2 chloroform-methanol-7M ammonium hydroxide (v/v). The total amount of DPC in the original sample was measured by counting an aliquot in ACS scintillation fluid. Values were expressed as cpm per 10^6 cells.

Fluorescence Microscopy

Confluent cell monolayers were established on 12 mm cover slips in MultiWell vessels. Cell preparations were washed once with PBS and stained for 2 to 5 minutes in the dark with phosphine 3R at a concentration of 1 μ g per ml of PBS. Cover slips were drained and placed on slides, with the cell side facing downward. Cells were examined for the presence of fluorescent inclusion bodies in the cytoplasm, typically observed in type II alveolar epithelial cells (9).

Transmission Electron Microscopy

Monolayer cultures grown to confluence in MEM supplemented with 10% FCS were fixed and processed by the method of Stratton (13) to observe multilamellar bodies. Cells were fixed with 3% glutaraldehyde in 0.1M cacodylate buffer, pH 7.3, for 3 hours at room temperature. Cells were then scraped directly into the fixative without rinsing and centrifuged at $400 \times g$ for 10 minutes. The cell pellet was resuspended in 1% tannic acid and 1% glutaraldehyde in 0.1M cacodylate buffer and treated for 2 hours at room temperature. Cells were then centrifuged, treated with 1% osmium tetroxide in 0.1M cacodylate buffer for 2 hours, and exposed to a series of acetone dehydration steps prior to embedding in Epon and sectioning.

RESULTS

Morphology of Hamster Tracheal Cells

After selective dissociation of tracheal tissue as previously indicated, specific cells attach to the vessel surface and appear to undergo extensive structural modulation during the first week of growth. Figure 1 is representative of the homogeneous cell population that arises during in vitro incubation. Note the considerable network of cytoplasmic granules (Fig. 1a). When the outer surfaces of individual cells are observed by interference

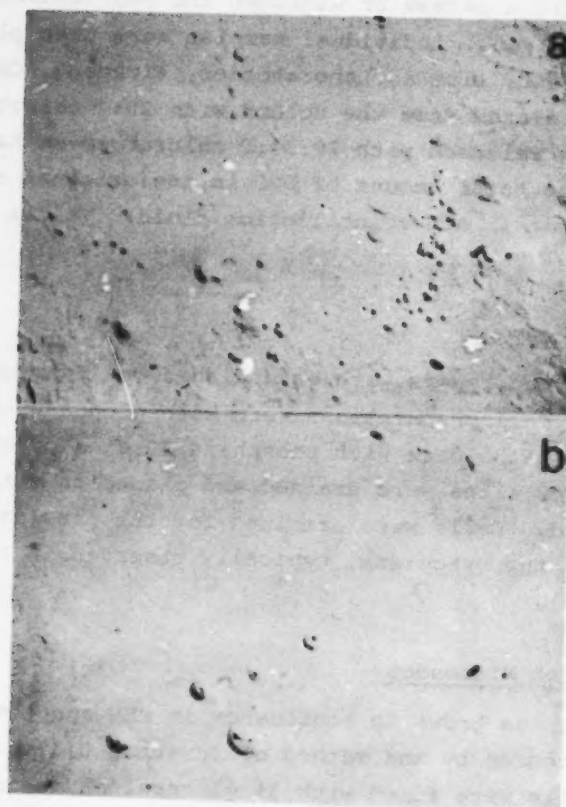


Figure 1. Cytoplasmic granules and secretory packets associated with monolayer cultures of hamster tracheal cells. Packets are visualized in cells by focusing in different planes using interference contrast microscopy, X850. In this set of photographs (a) represents the image in focal plane of the cytoplasm and (b) represents a view just above the cell surface.

contrast microscopy, prominent packets which apparently derive from active cell secretion are detected (Fig. 1b). These packets selectively react with histochemical stains for acidic mucins (6).

Glycoprotein Synthesis by Tracheal Cell Monolayers

Consistent with the histochemistry and tissue origin of these monolayer cultures, cells were readily capable of incorporating radioactive amino acid and sugar precursors into macromolecules which were then secreted into the medium. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of concentrated culture medium revealed a single radioactive peak with a molecular weight of about 18,500 daltons (Fig. 2b). Tracheal ring organ cultures

Table 1. Stimulation of Glycoprotein Secretion by Vitamin A

Test system ^a	Radioactivity (cpm) ^b per 50 µg cell protein	
	³ H	¹⁴ C
- Vitamin A	291.0 ± 16.4	2313.7 ± 192.5
+ Vitamin A	835.3 ± 120.6	4108.9 ± 286.2

^aCells were grown in MEM plus 10% FCS with or without vitamin A for 72 hours prior to addition of radioisotope for 24 hours.

^bBased upon incorporation of N-acetyl-D-galactosamine [acetyl-³H] and L-serine [U-¹⁴C] into a secreted glycoprotein. Values were calculated geometrically from cpm in the single radioactive peak on SDS-polyacrylamide gel, and are reported as the mean ± 1 standard deviation from the mean.

were radiolabeled under similar conditions, and an identical gel profile was obtained (Fig. 2a). The influence of vitamin A on the morphological and biochemical properties of this cell type was also examined. Absence of the vitamin from culture medium dramatically decreased both the number of cytoplasmic granules (Fig. 3) and glycoprotein secretion (Table 1) in monolayer cultures, correlating with the putative in vivo role of vitamin A (2).

Phospholipid Synthesis Among Various Cell Types

Cell monolayers of newborn hamster lung epithelium were monitored for their ability to synthesize lecithin and disaturated phosphatidylcholine. Most of the biochemical measurements were performed on cultures during the third to seventh in vitro passage. As seen in Table 2, these cells readily incorporated [³H]choline into phospholipid. Other animal cell cultures were also included in this study since it was important to establish the biologic specialization of the newborn lung epithelial cells. A significant range in the capacity of the various cultures to synthesize lecithin and disaturated phosphatidylcholine was observed. However, it was clear that the hamster lung cultures were most proficient in this bioassay.

Phospholipid Synthesis and Secretion by Normal and Transformed Lung Cells

During early cultivation (three to five passages) of hamster lung epithelial cells, increased ability to synthesize total phospholipid and disaturated phosphatidylcholine was demonstrated (Table 3). This observation appeared consistent with increases in both cell size and numbers of cyto-

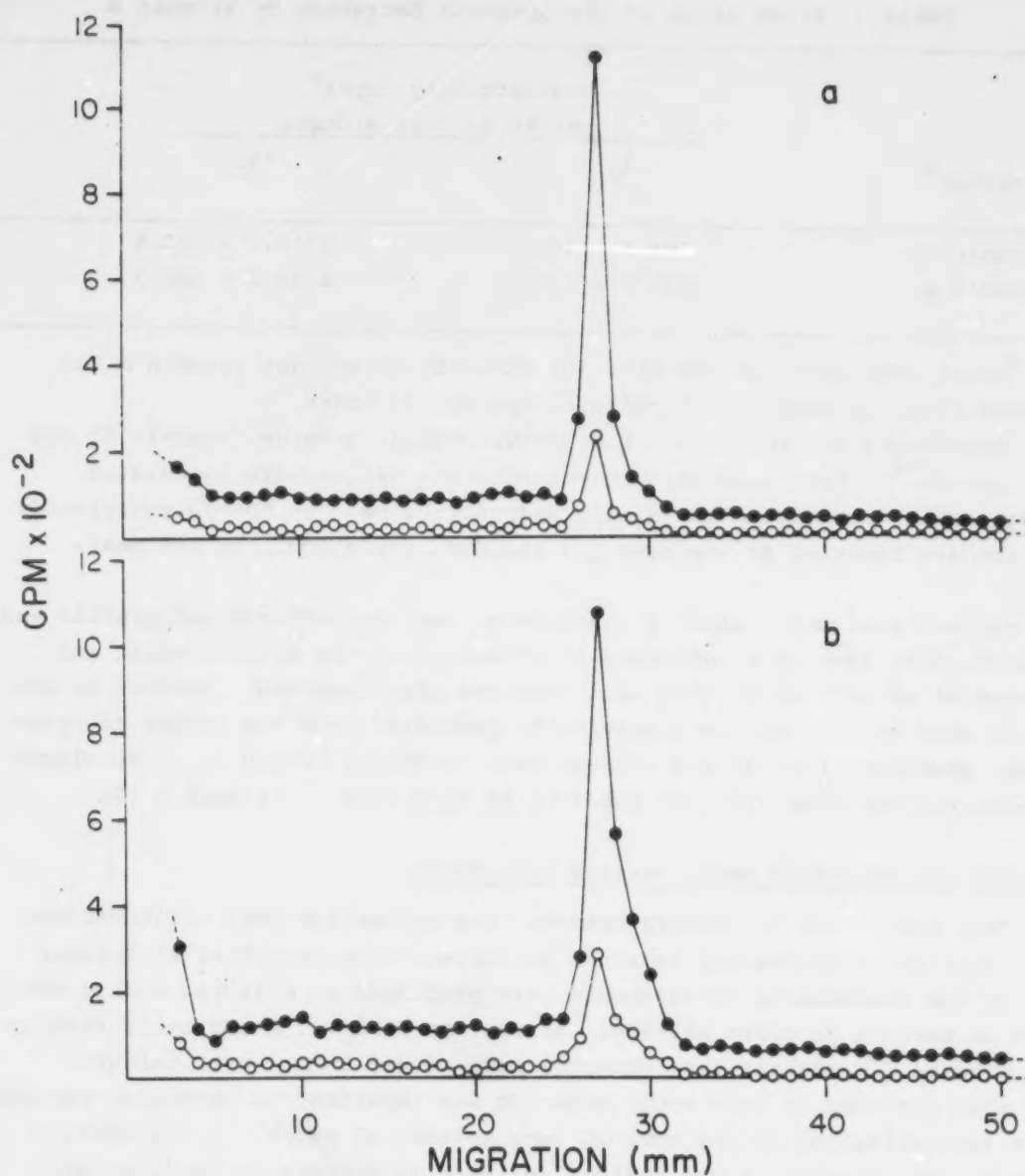


Figure 2. Glycoprotein synthesis by hamster tracheal rings and cultured tracheal cells. Profiles display the radioactivity from [¹⁴C]L-serine (●) and [³H]N-acetyl-D-galactosamine (○) in 1 mm slices of SDS-polyacrylamide gels. (a) Secretion collected from a pair of hamster tracheal rings in organ culture after 24 hours of incubation with radiolabeled precursors. (b) Secretion collected from a monolayer culture of tracheal cells incubated under similar conditions.

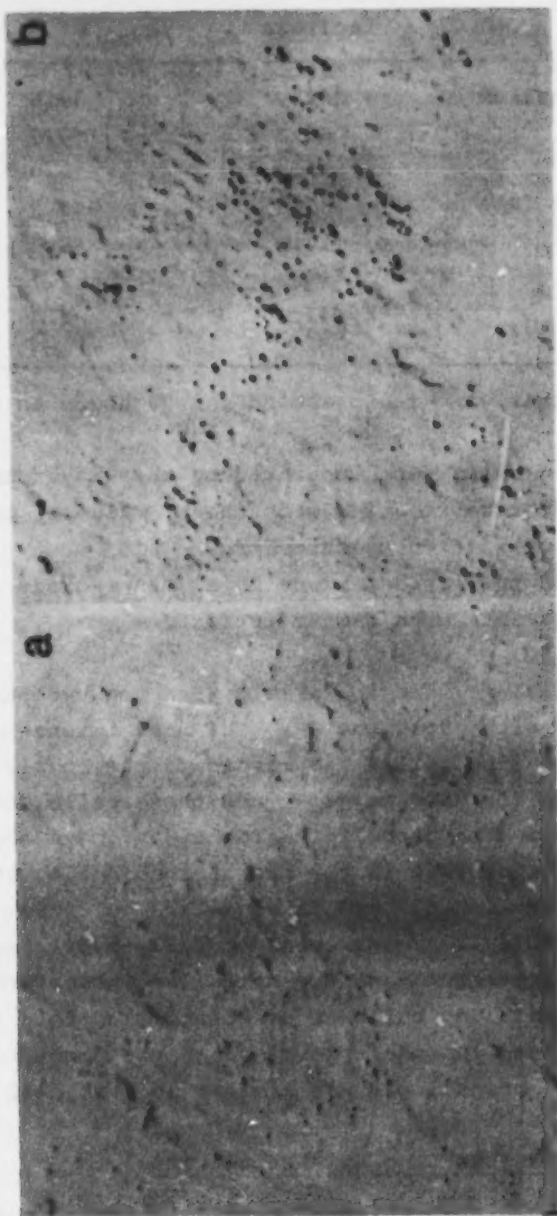


Figure 3. Effect of vitamin A on numbers of intracellular granules in cultured hamster tracheal cells. Interference contrast microscopy, X675. (a) Cells grown for 48 hours after removal of vitamin A from culture medium. (b) Cells maintained continuously in medium containing vitamin A (2 µg/ml).

Table 2. Incorporation of Choline Chloride [methyl-³H] into
Lecithin and Disaturated Phosphatidylcholine (DPC)
by Different Animal Cells

Cell type ^a	cpm x 10 ⁻⁴ per 10 ⁶ cells	
	Lecithin ^b	DPC ^c
Newborn hamster lung epithelium	114.0	19.6
Rat (L2)	68.2	11.6
Mouse (L25)	33.2	4.0
Cat (AKD)	30.0	3.3
Baby hamster kidney	24.9	4.7
Human A549	15.9	2.7
Chick embryo fibroblast	12.3	2.1

^aConfluent cell cultures were radiolabeled for 20 hours in serum-free MEM.

^bLipids extracted from cells were separated by thin-layer chromatography. The appropriate spot correlating with the R_f value of lecithin was removed and counted by scintillation spectrometry.

^cDPC was isolated from the lipid mixture by aluminum oxide chromatography after treatment of sample with osmium tetroxide.

plasmic granules. All cells examined in Table 2 with the exception of chick embryo fibroblasts stained with phosphine 3R. The most intense staining occurred in newborn hamster lung cells. As mentioned previously, naturally transformed clones arose during late passage, and these cells demonstrated a markedly reduced synthesis of phospholipid (Table 3). Note that the levels of lecithin and surfactant synthesis in the transformed cell cultures are similar to those of the established "type II alveolar" cultures (cat-AKD, human-A549) presented in Table 2. Comparisons of cell-associated and secreted disaturated phosphatidylcholine among the normal and transformed lung cell cultures revealed that normal lung cultures possessed a 10-fold higher capacity to synthesize and secrete phospholipid although the percentage of secreted surfactant to total phospholipid was similar in both cell types (Table 4).

Ultrastructure of Newborn Hamster Lung Epithelial Cells

One of the characteristic properties of type II alveolar cells is the existence of multilamellar bodies randomly distributed throughout the cytoplasm. These organelles serve as the source of surfactant, and alveolus function depends on their formation and secretion into the alveolar space. In

Table 3. Phospholipid Synthesis by Normal and Transformed
Newborn Hamster Lung Cells

Lung epithelium	Passage no.	cpm x 10 ⁻⁴ per 10 ⁶ cells	
		Lecithin	DPC
Normal	3	46.9	6.1
	4	57.0	11.6
	5	110.4	16.0
Transformed	9	35.0	4.2
	40	24.7	4.7

Experimental conditions are as in legend of Table 2.

Table 4. Synthesis and Secretion of DPC by Newborn
Hamster Lung Cells

Lung epithelium	% DPC ^a		Total cpm ^b
	Cell-associated	Cell-secreted	
Normal	71	29	13.0
Transformed	67	33	1.4

^aLipids were extracted separately from cells and culture fluid after 24 hours exposure to choline [methyl-³H] and processed as previously described.

^bTotal radioactivity represents the sum of DPC counts (x 10⁻⁴) in the cells and culture media fractions after aluminum oxide chromatography. All values are reported per 10⁶ cells.

Figure 4a, a classical membrane-limited lamellar body is observed in close association with Golgi vesicles, the latter probably being responsible for the generation of the lamellar structures. A lamellar body with its highly ordered structure appears at the cell surface (Fig. 4b) apparently after its release from the cytoplasm by a specialized type of exocytosis (11). Transformed lung cells derived from the hamster epithelial cultures also possessed similar multilamellar structures which were readily secreted. It is of interest that all other cell types (Table 2) examined by electron microscopy demonstrated cytoplasmic multilamellar bodies similar to those of the hamster lung epithelial cells.

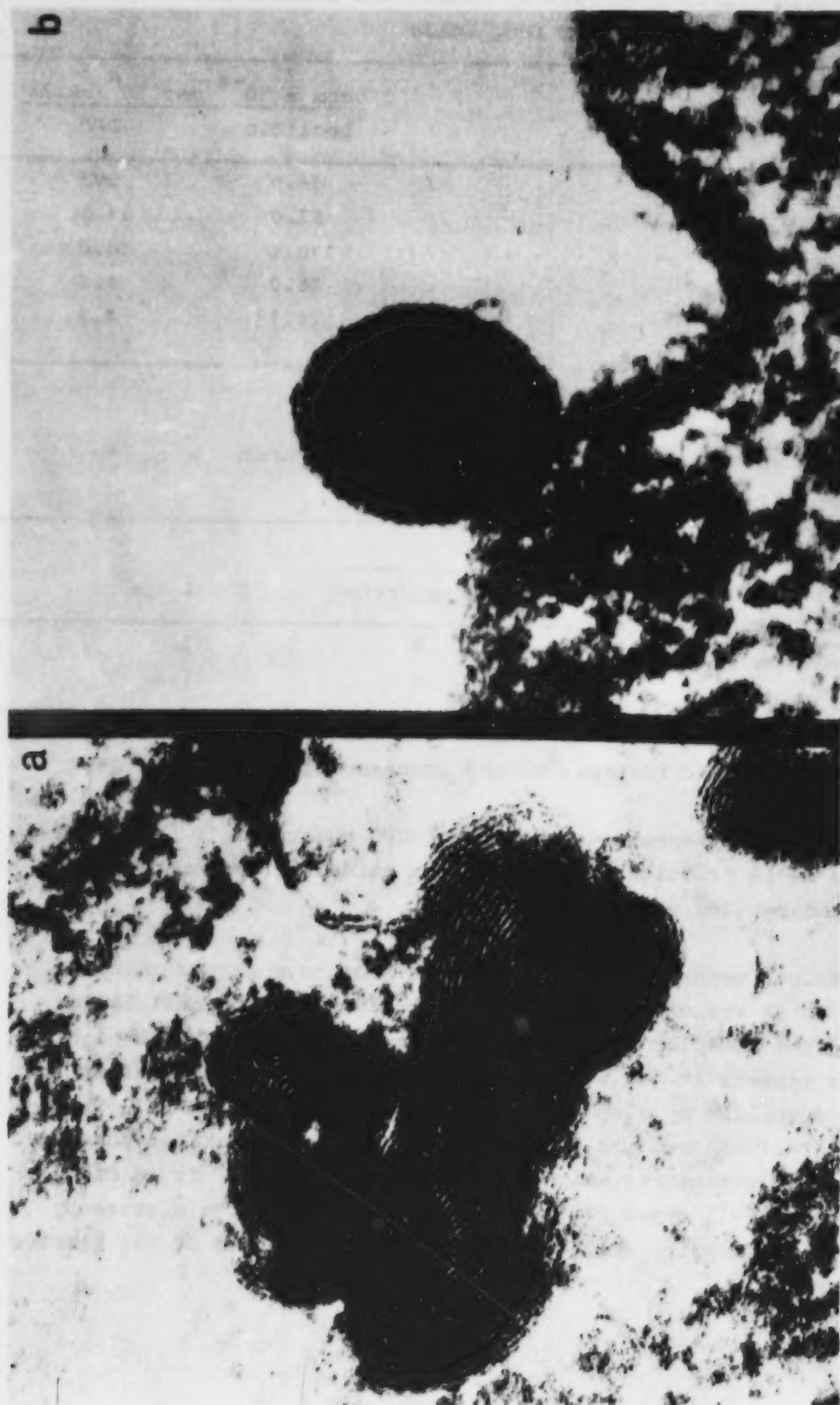


Figure 4. Transmission electron photomicrograph of newborn hamster lung epithelial cells during in vitro passage. In thin section (a), a mature multilamellar body is shown. Note the close association of Golgi vesicles (X225,500). In (b) a small compact multilamellar structure is associated with the cell surface, apparently originating via exocytosis (X280,500). Similar ultrastructure was observed in all hamster lung cell passages including transformed cell cultures.

DISCUSSION

Limited information is available concerning the biochemistry and metabolism of the mammalian respiratory tract which consists of many interacting cell types and related organs. The cellular heterogeneity and biologic complexity of the tracheobronchial epithelium have prevented the establishment in culture of pure populations of normal differentiated cells for monitoring respiratory function under various experimental conditions. Tissue slices, organ explants, primary and organotypic cultures, and selected cell purification procedures have offered useful models for examining pulmonary function. However, these systems do not provide homogeneous populations of epithelial cells that replicate in vitro with retained biochemical and ultrastructural specialization.

In this report we define selected properties of hamster respiratory cells readily passaged in vitro. Replicating monolayer cultures comprised of homogeneous populations of epithelial cells were obtained from trachea and lung tissues by a reliable technique previously described (6) and examined for their ability to synthesize and secrete mucus and surfactant, respectively. Evidence is presented to support the biosynthetic proficiency and uniqueness of these cell types.

Tracheal cell monolayers incorporated radioactive serine and N-acetyl-D-galactosamine into macromolecules with a molecular weight of approximately 18,500 daltons based upon SDS-polyacrylamide gel electrophoresis. Exposure of tracheal cell cultures to vitamin A stimulated cytoplasmic granule content and enhanced glycoprotein secretion. This information, along with previously described biochemical and histochemical data, suggests the potential application of this model system for investigating fundamental properties of mucus-secreting cells.

Characteristics of newborn lung epithelial cell monolayers described here reinforce their metabolic specialization. Synthesis of phospholipid and, more specifically, disaturated phosphatidylcholine, considered a major component of lung surface-active material, was greatest in these cultures when compared to a variety of other animal cell types. Electron microscopy of newborn hamster lung cells passaged in monolayer for four to eight weeks demonstrated the classical multilamellar bodies of type II alveolar cells. The proximity of these organelles to Golgi vesicles and the intense phosphine 3R staining of the cells supported their biologic identity with type II cells. The relationship between early passage lung cultures and late transformed cell clones revealed significant differences in capacity to synthesize surfactant. Transformed cells were much less biosynthetically active in terms of phosphatidylcholine synthesis although prominent cytoplasmic multilamellar bodies were still visualized.

Homogeneous cell populations of respiratory epithelium with retained capacity to synthesize and secrete mucus or surfactant can be experimentally manipulated, providing useful models for pulmonary research. Growth regulation and cellular differentiation can be measured in the presence of various stimuli or insults such as hormones and drugs, infectious agents, nutritional deficiencies, carcinogens, and pollutants. Chemical and physical properties of mucus glycoproteins and surfactant can be analyzed under a range of test conditions. Such studies should clarify the biologic competence of the tracheobronchial epithelium in maintaining pulmonary function and permit the identification of factors that alter the apparent normalcy of these respiratory cells.

ACKNOWLEDGMENTS

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DISCUSSION

DR. MOSSMAN: My question regards the tracheal epithelial cells. I wonder whether you were able to clone these cells. In other words, were pure populations of epithelial cells used in your experiments? Secondly, you mention in your abstract an actual transformation of these cells. What types of tumors did they give rise to in animals?

DR. BASEMAN: The first question concerns the ability to clone these cells. Most of the data presented are not based on cloned cell populations. Because the cloning process requires weeks before sufficient cell numbers are available for experimentation, we do not routinely clone. By the time we complete the cloning procedure, cells begin to senesce--i.e., the growth rate slows and individual cells enlarge. However, our specific cell isolation technique using a single hamster trachea results in the survival and growth of a small yet selective population of respiratory epithelial cells that exhibit morphologic homogeneity. When these cultured cells are compared to cloned cell populations, all cells appear identical based upon structural, biochemical, and histochemical criteria. Your second question concerns cell transformation. We have not isolated "transformed" cells from the trachea, but the data in the abstract refer to cells derived from hamster lung. In the latter case, we routinely clone cells because a greater variety of lung cells survive the cell isolation procedure in contrast to trachea-originated cultures. After prolonged culture of lung cells, the majority of cells exhibit characteristics of senescence. However, we observe small foci of cells that arise during the late passages. These cells are considered transformed because they demonstrate altered morphology, increased cell growth rate, and the property of continuous passage (over 110 transfers during an 18-month period). Also, these cells have a decreased capacity to synthesize surfactant although they do possess distinct cytoplasmic multilamellar structures. We have no evidence that these cells are neoplastic.

DR. MOSSMAN: The only reason I asked is that in our laboratory we isolated cells from the hamster trachea, and we also found that cells senesced. At this period of time, however, there were isolated populations which did multiply, and we were able to clone the cells. This was recorded as an annual meeting abstract for the Tissue Culture Association in In Vitro about four years ago.

DR. BASEMAN: I think that those cells differ from the tracheal cells that we are describing. We are not certain as to the absolute identity of our cells but believe that they possess specialized function in terms of mucus synthesis and secretion. These cells may originate from an "intermediate" cell which undergoes specific morphologic and physiologic changes in monolayer culture once removed from the environmental integrity of the hamster trachea.

DR. HU: I understand. Some time ago you discussed with me the technique of isolating respiratory cells.

DR. BASEMAN: Yes. After about a year of considerable effort, we consistently generate these epithelial cell cultures from the hamster trachea. Why have others been less successful? I believe that the cell isolation technique that we have developed which allows the selective dissociation and survival of specific tracheal cells prior to monolayer culture is the key.

DR. DAVIS: I agree with you that this is a useful method for studying respiratory cell physiology. In fact, we are investigating a similar area. What I do not know is what the criteria should be for defining mucus-secreting cells. Have you measured ion content of the cells?

DR. BASEMAN: These monolayer cultures of tracheal cells appear to synthesize mucus selectively when compared to other available cell types. In terms of ion flux, one of the problems is that we really do not know the exact criteria for designating a cell "specialized." We think that when a cell selectively synthesizes a macromolecule that appears distinct biochemically and histochemically and that this function is under regulatory control as indicated by vitamin A responsiveness while other cell types lack this biosynthetic capability, then the cell should be classified as specialized.

Retinoid Metabolism and Mode of Action

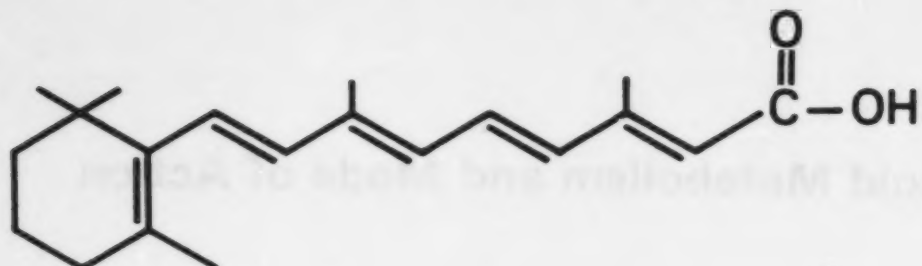
L. M. De Luca, W. Sasak, S. Adamo, P. V. Bhat,
I. Akalovsky, C. S. Silverman-Jones, and N. Maestri
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Epidemiological studies indicate that the majority of human cancers are caused by exposure to environmental carcinogens (13). These can be inhaled, ingested with food, or absorbed through the skin and first come in contact with the epithelial surfaces of the body, which have a greater metabolizing activity than stromal fibroblasts. Related to this greater metabolic activity for chemical carcinogens is the fact that approximately 80% of human cancer originates in epithelial tissues (11).

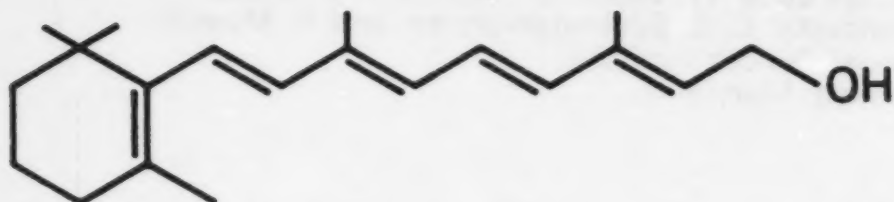
Although exposure to carcinogens is essential for the neoplastic event to occur, host factors are very important modulators of the oncogenic response. These factors may be genetic or acquired. An example of genetic factors is the levels of carcinogen-activating enzymes, which appear to vary profoundly among different individuals (11). An example of acquired factors is vitamin A.

The interest in vitamin A and its derivatives, the retinoids (22), has derived from old and fundamental observations that the vitamin preserves the integrity of epithelial tissue (for a review see ref. 7). Feeding a vitamin A-deficient diet to hamsters causes replacement of the normal mucociliary epithelium of the trachea by squamous metaplastic and keratinized cells (7, 22). A similar response may also be caused by chemical carcinogens or mechanical injury. These conditions may be corrected by administering vitamin A either to the whole animal or to the organ in culture (5, 14).

Because of these findings a flurry of activity in the area of prevention



all-trans RETINOIC ACID



all-trans RETINOL

Figure 1. Structures of retinoic acid and retinol.

of chemical carcinogenesis by retinoids has occurred in recent years with the scope of finding a potent and non-toxic retinoid, which might prevent neoplastic transformation in selected human populations at high risk of developing cancer.

Retinoids are also active in the alleviation of a variety of skin diseases, including actinic keratosis and basal cell carcinomas (16, 19).

Inasmuch as derivatives of retinoic acid (Fig. 1) are not stored in the liver and reach the target site in larger amounts than retinol (Fig. 1), they have been used preferentially in preventive and therapeutic studies (7, 16, 22).

The proposed concept is that, by increasing the concentration of the retinoid in the target tissue, one enhances the potential of the tissue to maintain the normal phenotype, thereby counteracting the sequel of tissue alterations eventually leading to the neoplastic transformation caused by carcinogens.

Although clearly such preventive studies do not require a full knowledge of the biochemical mechanisms of action of the retinoids, such knowledge may be crucial in understanding how the phenotypic expression of epithelial tissues is maintained.

RESULTS

The Tracheal Epithelium

The integrity of the tracheal epithelium is lost in vitamin A deficiency. A study of the morphology of tracheal explants from vitamin A-depleted hamsters and rats cultured in a vitamin A-depleted medium shows that the normal mucociliary epithelium is replaced by a squamous metaplastic epithelium which keratinizes and sheds keratin into the medium (5, 14).

As in other epithelial tissues, a major biochemical defect in vitamin A deficiency in the respiratory mucosa occurs at the level of biosynthesis of specific glycoproteins (for a review see ref. 6). Such alterations in glycoprotein biosynthesis may have important consequences for the membrane. The carbohydrate moieties of glycoproteins appear to act as "lectins" in that they bind specifically to sites which recognize their terminal sugar at the non-reducing end of the macromolecule (21).

Ashwell and Morrell and their collaborators (4) have shown that glycoproteins are readily removed from the blood stream of the rat when their terminal sialic acid residues are removed and the penultimate sugar, galactose, is exposed to allow recognition by a molecule at the surface of the hepatocyte which sequesters the circulatory glycoprotein into the hepatocyte (4) and away from circulation. Similar recognition properties have been suggested by Roseman (20) to mediate intercellular interactions and the phenomenon of cellular adhesion.

There is now a vast body of work to indicate that vitamin A is directly involved in controlling the biosynthesis of specific glycoproteins in all epithelial tissues so far studied (6). Most of this work has been conducted in the whole animal, using radioactively labeled monosaccharides as precursors of glycoproteins. More recent work has confirmed these findings in tissue culture systems. Particularly, cultures of mouse epidermal cells have been shown to respond to retinylpalmitate by a remarkable (5- to 11-fold) increase in the incorporation of galactose, mannose, and glucosamine into epidermal glycoproteins (3).

Similar results were obtained in organ cultures of corneal epithelial cells from the eyes of rats. In this system it was shown that the corneal epithelium responds to culturing in the presence of retinol or retinoic acid by a selective increase in the incorporation of monosaccharides and without effect on the incorporation of leucine (12). The incorporation of glucosamine into high molecular weight glycoproteins (greater than 200,000 daltons) was specifically enhanced by vitamin A, either in organ culture or *in vivo* (12). Thus, vitamin A seems to control properties of biological membranes by controlling the biosynthesis of specific glycoproteins of the membrane.

Incorporation of [³H]benzo(a)pyrene into Normal and Vitamin A-deficient Hamster Tracheas

A faulty membrane may allow a greater amount of environmental pollutants inside the cell.

In a series of experiments we set out to measure the amount of [³H]benzo(a)pyrene incorporated into negatively charged lipids of hamster tracheas from normal and vitamin A-depleted animals.

Tracheas (10 per group) were incubated in culture medium L-15 for 30 minutes at 37°C. The medium contained either 5 μ Ci of [³H]3,4 benzo(a)pyrene (specific radioactivity 11 Ci/mMole), 5 μ Ci of [¹⁵-³H]retinol (specific radioactivity 1.25 μ Ci/mMole) or 5 μ Ci of [¹⁴C]mannose (50 μ Ci/mMole). Retinol and mannose were used to study the specificity of the effect on benzo(a)pyrene. The incorporation of these radioactive precursors into membrane phospholipids was measured by chromatography of the lipidic extract (chloroform/methanol 2/1) on columns (0.75 x 45 cm) of DEAE-cellulose acetate. The free precursors were eluted off the column with about 400 ml of 99% methanol. The radioactivity associated with phospholipids was eluted with a gradient of 100 ml of 99% methanol to 100 ml of 100 mM ammonium acetate.

Table 1 shows that the amount of each of the three precursors incorporated into negatively charged lipids of the tracheas from vitamin A-depleted hamsters was several fold greater than in normal tracheas.

The vitamin A-deficient tracheas incorporated 3.9 times as much benzo(a)pyrene into phospholipids as their normal counterparts (Table 1). This finding is in agreement with previous results describing a larger amount of radioactive benzo(a)pyrene bound to the DNA of vitamin A-deficient tracheas (10).

Table 1. Incorporation of [³H]Benzo(a)pyrene, [³H]Retinol and [¹⁴C]Mannose into Negatively Charged Lipids of Normal and Vitamin A-deficient Hamster Tracheas

	<u>μMoles incorporated in 30 minutes at 37°C</u>		
	Normal	Deficient	D/N
Benzo(a)pyrene	1.95×10^{-7}	7.66×10^{-7}	3.92
Retinol	16.9×10^{-7}	89.5×10^{-7}	5.29
Mannose	1.83×10^{-5}	5.09×10^{-5}	2.78

Conditions of incubation and processing of the lipid extracts are described in the text. D/N stands for deficient/normal.

However, retinol and the monosaccharide mannose were also incorporated in larger amounts in the phospholipidic fraction of the deficient organs (Table 1), suggesting that general permeability characteristics of the deficient epithelium are profoundly altered in vitamin A deficiency. It is therefore reasonable to consider that the epithelial uptake of toxic substances from the environment may be modulated by the nutritional status of the animal.

The Involvement of Retinoids in Adhesion

Vitamin A also affects the adhesive properties of cells. Glycoproteins, (25), glycolipids, and glycosyltransferases (20) have been implicated as the molecular determinants of adhesion. Inasmuch as retinol and retinoic acid are essential constituents of membranes and modulators of the biosynthesis of specific glycoproteins, it was of interest to determine whether they could influence adhesion.

The system of choice was the spontaneously transformed mouse fibroblasts (Balb/c 3T12-3 cells) because these cells display very poor adhesive properties in culture and can be lifted from the culture dish surface in an EDTA-mediated detachment assay (1). The cells appear round and display the typical morphology of transformed cells with very poor adhesion to each other (Fig. 2A). However, when cultured in the presence of 3.3×10^{-6} M retinol or retinoic acid, they acquire morphological characteristics of "normal" cells (Fig. 2B). Moreover, they remain attached to the plate, when treated with EDTA, under conditions which lift the untreated cells. Eventually, retinoid-treated cells also come off the plate, but only after prolonged EDTA treatment. Interestingly, saturation density was not affected markedly by retinoid treatment and the more adhesive, retinoid-treated cells had the same plating efficiency as the untreated 3T12 cells in dense (90%) and sparse (20%) culture conditions.

Inasmuch as this assay might be useful to measure retinoid-activity, structure-activity relationship was investigated in this system. Retinol, retinoic acid, and their 5,6-epoxyderivatives were the most active compounds (2, 8). Derivatives of retinoic acid with activity in the hamster trachea differentiation system (22) were also active in increasing adhesion. Derivatives of retinol and retinoic acid, which were devoid of any vitamin A activity in other systems, were also inactive in increasing adhesion (2, 8). The free carboxyl group of retinoic acid was necessary for activity, since esterification or amide formation led to inactive compounds.

In conclusion, it appears that this newly found biological activity of retinoids in increasing adhesion of 3T12 cells correlates well with their vitamin A activity in other biological systems; it also offers an easily accessible and relatively fast assay for vitamin A activity.

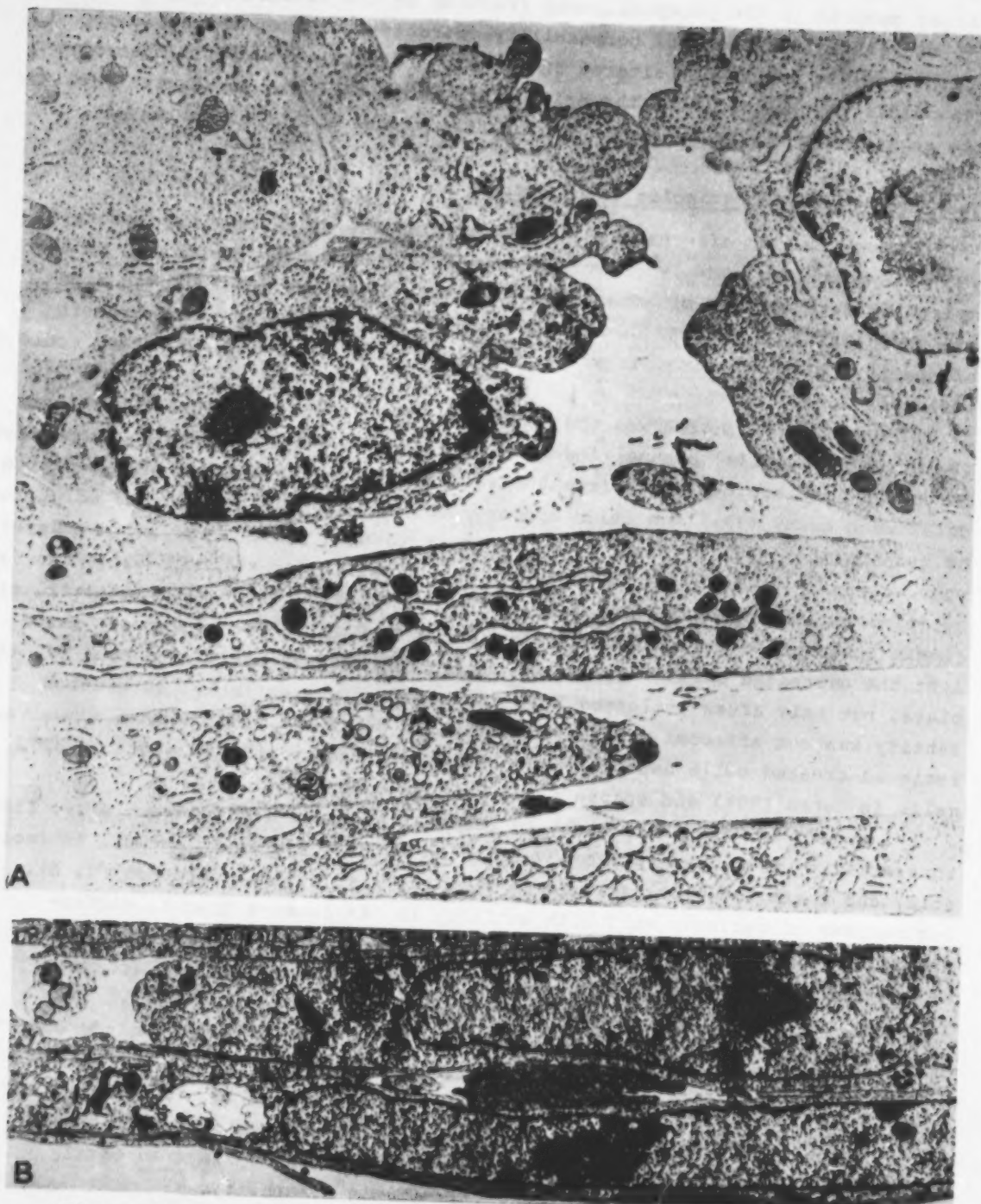


Figure 2. Transmission electron microscopy of control (A) and retinoic acid-treated (B) 3T12 cells (X3,500). Cells were seeded at 10,000 per cm^2 and grown in the presence or absence of 3.3×10^{-6} M retinoic acid for six days.

An investigation of the biosynthesis of glycoproteins in this system of transformed cells is now under study. The incorporation of [2-³H]mannose into glycoprotein is greatly enhanced by retinoid treatment at the same time as the increase in adhesion is observed. Particularly, the biosynthesis of a mannose-containing glycoprotein of molecular weight 180,000 daltons (gp180) was found to be highly stimulated by retinoids, whereas not much effect was noticed on the biosynthesis or the iodination of the extracellular fibronectins, which have been implicated in the phenomenon of intercellular and cell to substrate adhesion (25).

In 1970 we first suggested that vitamin A controls membrane function by its molecular involvement in the biosynthesis of glycoproteins (6). We proposed that the phosphorylated form of vitamin A carries glycosyl residues across the membrane bilayer to make them available for glycosylation of glycoproteins. This hypothesis has received considerable attention after the demonstration that phosphorylated vitamin A (retinylphosphate) is a component of epithelial membranes (6). The biosynthesis of retinylphosphate (6) has been demonstrated in vivo in rat liver and intestine (6, 15) and in vitro in cultures of rat intestinal cells (9) and mouse epidermis (3).

Figure 3 illustrates the proposed scheme for the action of mannosyl-retinylphosphate in the membrane. The molecule is shown with its hydrophobic cyclohexene ring embedded in the lipid bilayer and the polar end in the hydrophilic area in the all-trans configuration. The trans-9-cis isomerization allows crossing of the bilayer with the mannosyl moiety now in the "inside" of the membrane. Such trans-cis isomerization is postulated here to occur via an isomerase enzyme. The 9-cis mannosylretinylphosphate would then function as a donor of mannose to acceptor glycoproteins through the action of glycosyl transferases.

CONCLUSIONS

In addition to the role of vitamin A in the visual cycle as the chromophore of rhodopsin (17, 24), evidence is now emerging for a new biochemical function for the vitamin as a carrier of mannosyl residues for the biosynthesis of glycoproteins (6). Such a carrier role appears to be essential for the biosynthesis of mannose-containing glycoproteins, as suggested by studies in deficiency and excess vitamin A. There is in fact a 70% reduction in the amount of mannose bound to glycoproteins of the hepatocyte membrane in vitamin A deficiency and a 610% increase in the incorporation of mannose in excess vitamin A.

It is thus suggested that, if such glycoproteins are necessary to regulate membrane functions, the diverse morphological and functional manifestations of the action of vitamin A [i.e., mucociliary function in respi-

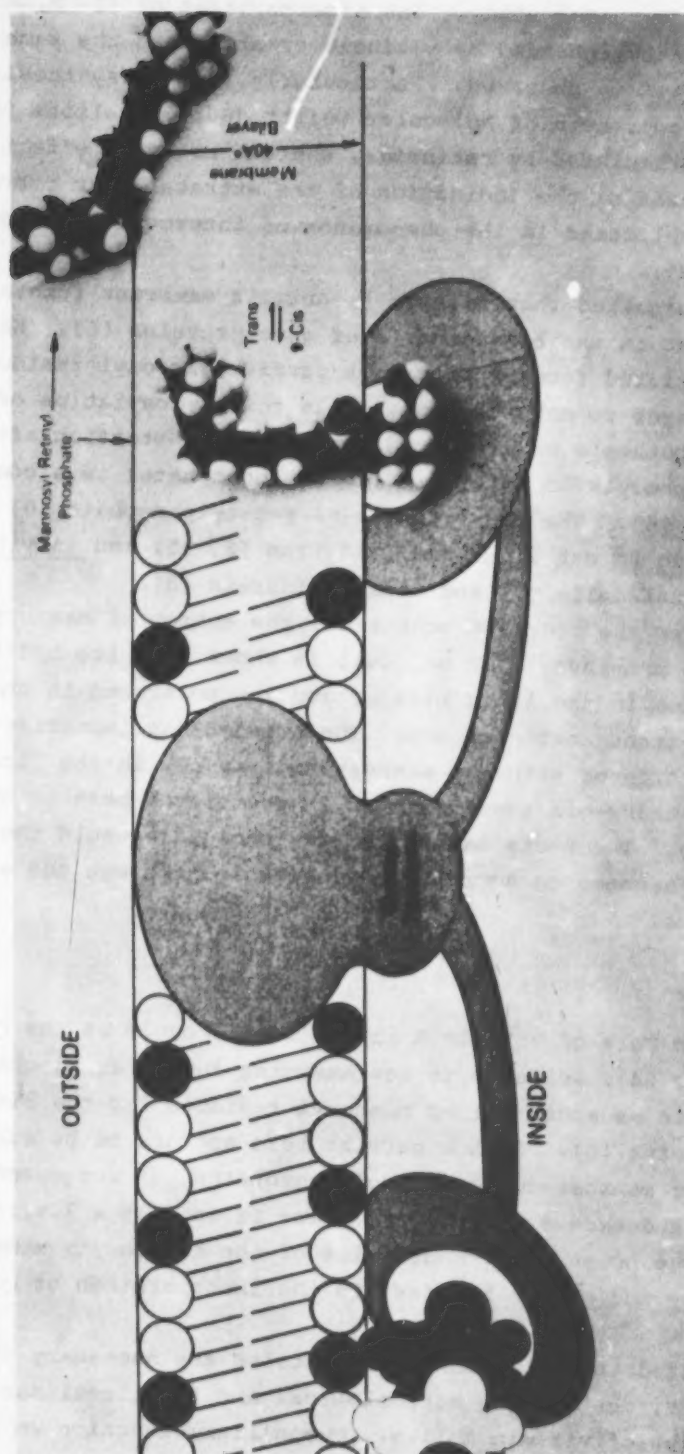


Figure 3. Working scheme for a trans-cis membrane-crossing action of vitamin A in glycosyl transfer reactions. The lipid constituents of the membrane bilayer are shown with their polar head groups on the exterior and the hydrophobic fatty acid chains in the interior of the bilayer. The trans to 9-cis isomerization permits the crossing of about 40 angstroms by the mannosyl residue attached to retinyl phosphate. The 9-cis molecule would then serve as a substrate for the mannosyl transferase.

ratory epithelium (6), adhesive properties in fibroblasts (1, 2, 8), hormonal responses in the reproductive systems (23), etc.] depend on the particular tissue. Moreover, it appears that, in addition to retinol, a metabolite of retinoic acid undergoes the same fate of phosphorylation and glycosylation (6), thus functioning as vitamin A.

It is tempting to suggest that the function of the binding proteins for retinol and retinoic acid is a protective one (18) against oxidation and metabolism, prior to insertion of the vitamin in the hydrophobic environment of the membrane bilayer, where it functions in the phosphorylated form.

Inasmuch as the vitamin maintains and pollutants perturb the normal phenotype of the mucociliary epithelium, it would be of interest to study whether toxic agents, such as ozone, interfere with the vitamin A-dependent biochemical processes of epithelial tissues by destroying the retinol, retinyl esters, and retinylphosphate present in these tissues. It would also be of interest to measure whether an increased intake of nutritional factors such as vitamin A would have a protective effect against the action of toxic substances in the respiratory epithelium exposed to the toxic agent.

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DISCUSSION

PARTICIPANT: Is there any evidence that oxidation of the retinoic acid or the retinol would decrease its activity?

DR. DE LUCA: Yes, definitely. Even the 5,6 epoxidation of retinol and retinoic acid will produce compounds which in vivo have been reported by Dr. Cama to be less active than the retinoic acid or retinol. If you oxidize the chain, I would say that, in general, oxidation leads to destruction or loss of activity.

DR. REID: What other sugars are carried, or is it only the mannose?

DR. DE LUCA: Yes, we have worked mostly with mannose. But Dr. Peterson and Dr. Wolf have done some work in recent years with galactose. So, it appears that both mannose and galactose are involved.

DR. NETTESHEIM: There are two reports from the literature that I am aware of concerning the inhibition of in vitro transformation by retinoids with x irradiation and with chemical carcinogens. Do you have any suggestions to make? What the mechanism involved might be? Is it possible that since these people are really looking at the phenotypic change, they are looking at a similar phenomenon that's related to the adhesion phenomenon, or do you think that it is really something that has to do with inhibition of neoplastic transformation?

DR. DE LUCA: You're asking me, do we know what neoplasia is all about? I don't know. It's a very difficult question.

Session VI:
Pharmacological Modulation

The Lung Mast Cell: Its Physiology and Potential Relevance to Defense of the Lung

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The mast cell, with its specific immunologic receptors, is positioned at portals of entry of potentially toxic or noxious exogenous material. In the lung the mast cell is located free in the bronchial lumen, in the bronchial mucosa in intra-epithelial locations, as well as in deeper perivenular collections (35, 37). As it is present prior to entry of noxious agents and is thus freed from the requirements of mobilization and localization, the mast cell may be the sentinel cell for induction of local inflammatory responses. The development of inflammation by mast cells is consequent to the release of its content of potent biologic mediators as well as by its capacity to generate, de novo, active biologic materials from the local microenvironment. The knowledge that mediators are active in vivo follows from the in vitro definition of their biologic activities which relate to the known pathophysiology of inflammation. The inflammatory processes induced by mast cell mediators are both acute and chronic and are subject to both positive and negative feedback controls inherent in the properties of the mediators themselves. The purpose of this review is to delineate the physiology of the mast cell, the mediators generated upon mast cell activation, and the regulation of mast cell-dependent inflammatory events.

*Dr. Wasserman is a recipient of an Allergic Diseases Academic Award (AI-00254) from the National Institutes of Health.

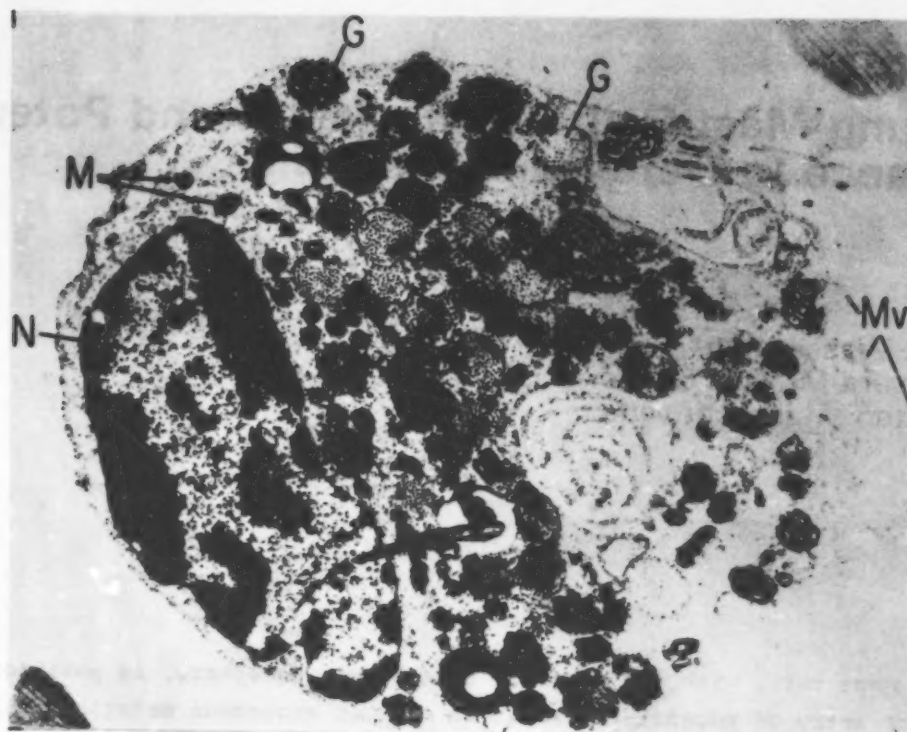


Figure 1. Electron micrograph of isolated human lung mast cell. Nucleus (N), membrane microvilli (MV), granule (G).

MAST CELLS

Mast cells are present in human lung at concentrations of $1-7 \times 10^6$ cells/g lung tissue (30). Each mast cell possesses, in addition to the ubiquitous subcellular organelles essential for all cell function, several hundred metachromatically staining granules, each surrounded by a bilayer membrane. Lung mast cell granules possess a definite subgranular architecture (Fig. 1) of unknown functional significance. The mast cell membrane is ruffled and possesses 50-300,000 receptors for IgE (15). IgE molecules belong to an immunoglobulin class defined by its activity in mediating immediate hypersensitivity reactions and by characteristic physicochemical properties. It is comprised of two heavy and light chains linked by disulfide bridges with a total molecular weight (m.w.) of 190,000. It is heat labile, but does not form precipitates with specific antigen nor does it fix complement. The serum concentration of IgE is under genetic control, being higher in atopic than in normal individuals. Papain digests IgE into three fragments, two of which (Fab) bind specific antigen and one (Fc) which binds to the specific receptors on the mast cell surface. Receptors for the anaphylatoxins, C3a and

CSa, fragments of the third and fifth components of complement, respectively, have been attributed to the mast cell upon functional criteria. In addition, mast cells may be degranulated by non-immunologic stimuli. Thus enzymes such as chymotrypsin, phospholipases A and C and sialidase, ionophores, polycationic amines and proteins, radio-contrast media, and opiates may all effect mast cell degranulation. While it is generally assumed that atopic individuals exhibit their antigen-induced symptoms as a result of IgE-dependent mast cell activation, the demonstration of non-IgE-mediated mechanisms for mast cell mediator release does not diminish the primacy of IgE in allergy but rather yields additional information on potential mechanisms for recruitment of mediators.

ACTIVATION AND DEGRANULATION OF MAST CELLS

The IgE-dependent degranulation of mast cells is initiated by the bridging of pairs of cell-bound IgE by specific antigen and terminates in two minutes. Bridging results in an alteration of the cell membrane which probably activates a surface membrane esterase. The cell membrane perturbation thus initiated is also associated with increased energy-dependent calcium entry into the cell. The calcium requirement for degranulation is related to the activation of the membrane serine esterase, and perhaps also to requirements for the expression of phospholipase activity, microfilament contraction, or membrane fusion. Degranulation also requires glucose and an intact glycolytic pathway. Finally, both ATP and calcium are required by a calcium-dependent ATPase which probably activates contractile proteins. Following the ordered completion of these processes, the perigranular membranes fuse with each other and with the cell membrane, and the granules are extruded (Fig. 2). Degranulation may be modulated at several steps in its sequence by endogenous or exogenous agents. Thus, elevations in intracellular levels of cyclic adenosine 3'5'-monophosphate (cAMP), which may be induced by prostaglandins, histamine, and β -adrenergic agents, inhibit degranulation (16). Conversely, α -adrenergic- or prostaglandin-induced falls in cAMP or rises in intracellular cyclic guanosine 3'5'-monophosphate (cGMP) following interaction with cholinergic stimuli or histamine may enhance degranulation (16). The opposing actions of the cyclic nucleotides and the bidirectional control of degranulation by cAMP are felt to represent competitive effects upon a putative protein kinase which might regulate the state of cytoskeletal assembly. The supposition that cytoskeletal elements are important in human mast cell degranulation is supported by the inhibitory effects upon degranulation of colchicine and by the augmentation induced by cytochalasin B (10, 27).

Table 1. Mast Cell-Dependent Mediators

Mediator	Structural Characteristics	Function	Inhibition	Inactivation
I. BRONCHOSPASTIC AND VASOACTIVE ACTIVITY				
Histamine (preformed)	β -imidazolyl-ethylamine MW 111	Contraction of smooth muscle, increase of vascular permeability, stimulation of suppressor T-lymphocytes, generation of prostaglandins, enhancement (H_1) or inhibition (H_2) of chemotaxis, elevation of cAMP (H_2) and cGMP (H_1)	H_1 classical H_2 thiourea	Histaminase (diamine oxidase) or Histamine N-methyl transferase
SRS-A (newly generated)	Acid hydrophilic sulfur containing lipid (?) MW $\sim$ 400	Contraction of smooth muscle, increased vascular permeability, synergistic with histamine, generation of prostaglandins	FPL-55712	Arylsulfatases A and B
Serotonin (preformed)	5-OH-tryptamine MW 182	Contraction of some smooth muscle, increased vascular permeability	Hydroxyzine Cyproheptadine Lysergic acid	Monoamine oxidase

Table 1 Continued

Mediator	Structural Characteristics	Function	Inhibition	Inactivation
PAF(s) (newly generated)	Lipid-like MW 400-1000	Release of platelet amines, platelet aggregation, sequestration of platelets	Unknown	Phospholipases
Arachidonic acid metabolites PGD, PGE, PGI, PGF _{2α} , HETE	C ₂₀ fatty acids (HHT C ₁₇ fatty acid)	Contract smooth muscle (PGD ₂ , PGF _{2α} , TxA, PGG ₂ , PGH ₂) Relax smooth muscle (PGE ₂) Elevate cyclic AMP (PGE, PGD, PGI), elevate cyclic GMP (PGF _{2α} , PGG ₂) dose-dependent chemotactic attraction of eosinophils or neutrophils (HETE or HHT)	ETYA (cyclooxygenase lipooxygenase) Nonsteroidal anti-inflammatory agents (cyclooxygenase)	Several specific prostaglandin-modifying enzymes
II. CHEMOTACTIC ACTIVITY				
ECF-A (preformed)	Val/Ala-Gly-Ser-Glu MW 360-390	Chemotactic attraction and deactivation of eosinophils and neutrophils	Gly-Ser-Glu Val-Gly-Ser Ala-Gly-Ser	Amino-peptidase Carboxypeptidase A

Table 1 Continued

Mediator	Structural Characteristics	Function	Inhibition	Inactivation
ECF-oligo-peptides (preformed)	Peptides MW 1300-2500	Chemotactic attraction and deactivation of eosinophils and neutrophils	Unknown	Unknown
NCF (preformed)	Neutral protein MW > 750,000	Chemotactic attraction and deactivation of neutrophils	Unknown	Unknown
Lipid chemo-tactic factors (newly generated)	? HHT ? HETE ? other lipids	Chemotactic attraction of neutrophils and eosinophils Chemokinesis of neutrophils Deactivation of neutrophils	Unknown	Unknown
Histamine (preformed)	β -imidazolyl-ethylamine MW = 111	H ₁ chemo-tactic and chemokinetic activation of eosinophils H ₂ chemo-tactic and chemokinetic inhibition of neutrophils and eosinophils	H ₁ classical H ₂ thiourea	Histaminase (diamine oxidase) or Histaminase N-methyl transferase

Table 1 Continued

Mediator	Structural Characteristics	Function	Inhibition	Inactivation
III. STRUCTURAL COMPONENTS				
Heparin (preformed)	Proteoglycan MW $\approx$ 60,000	Anticoagulation Antithrombin III interaction Inhibition of complement activation	Protamine	Heparinase
Chondroitin 4 and 6 sul- fate (preformed)	Proteoglycan	Platelet factor IV interaction	Unknown	Chondroitin- nase AC
Dermatan sulfate (preformed)	Proteoglycan	Unknown	Unknown	Chondroitin- nase ABC
IV. ENZYMES				
Chymase (preformed)	Protein MW = 29,000	Proteolysis with chymo- tryptic speci- ficity	Serotonin Heparin Chymotrypsin inhibitors	Unknown
Arylsulfa- tase (pre- formed)	Protein MW = 100,000 (A) 60,000 (B)	Hydrolysis of SRS-A and various sul- fate esters	PO ₄ , SO ₄ Product, substrate	Unknown
N-acetyl- β - D-glucos- aminidase (preformed)	Protein MW = 158,000	Cleavage of glucosamine residues	Product	Unknown

Table 1 Continued

Mediator	Structural Characteristics	Function	Inhibition	Inactivation
Basophil (lung)	Protein MW $\approx$ 400,000	Proteolysis with tryptic specificity	Trypsin inhibitors	Unknown
Kallikrein of anaphylaxis (preformed)		Cleavage of kinin from kininogen Cleavage of Hageman factor		
β -glucuronidase	Protein MW $\approx$ 300,000	Cleavage of glucuronide conjugates	Product	Unknown

histamine N-methyl transferase) (42). The pulmonary effects of histamine are expressed as both direct and reflex constriction of both large and small airways smooth muscle, thereby increasing airway resistance and decreasing compliance (20). It also dilates small radicles of the pulmonary vascular tree and increases the distance between endothelial cells of the venules, thereby increasing the potential for transudation of serum and for extravasation of leukocytes.

The biologic activities of histamine are expressed by its interaction with either of two specific classes of receptors on target cells. Those receptors designated H_1 predominate in skin and smooth muscle and are blocked by classic antihistamines, while H_2 receptors are selectively blocked by a group of compounds, including the thiourea derivatives, buramimide, metiamide, and cimetidine (4). Pulmonary bronchoconstriction, vasodilation, and increased cGMP are H_1 effects, while H_2 effects include inhibition of both human lymphocyte-mediated cytotoxicity and IgE-mediated histamine release due to elevation in cAMP content. Histamine also inhibits chemotaxis through H_2 receptors, presumably also by stimulating adenylate cyclase and increasing cAMP.

Slow Reacting Substance of Anaphylaxis (SRS-A). Although SRS-A remains structurally undefined, it is likely an unsaturated acidic sulfur-containing lipid of 300-500 daltons (28). It is active as a constrictor of peripheral airways to a much greater extent than of central airways (9) and causes vasodilation. Human lung tissue, dispersed pulmonary cells, and nasal polyps

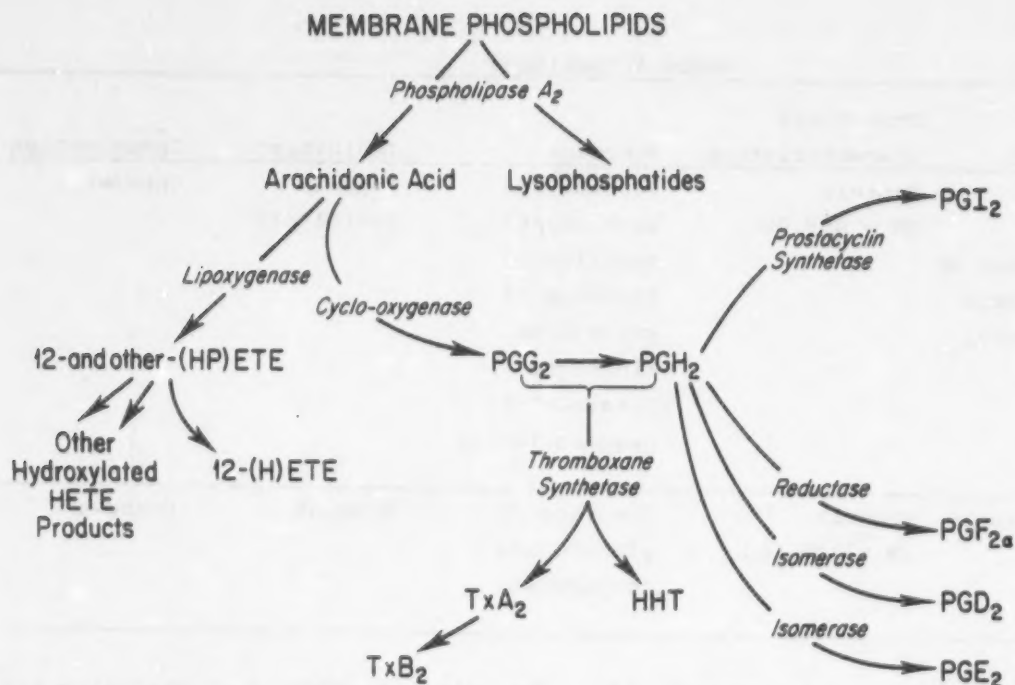


Figure 3. Pathways of generation of oxidative metabolites of arachidonic acid. Tx = thromboxane, PG = prostaglandin, HETE = hydroxy eicosatetraenoic acid, HHT = 12-L-hydroxy-5,8,10 heptadecatrienoic acid. [Modified from Lewis and Wasserman: In: The Science and Practice of Clinical Medicine: Rheumatology (Cohen A, ed). New York, Grune & Stratton (In press)]

generate SRS-A during IgE-dependent immunologic reactions. Decreasing amounts of SRS-A are generated by increasingly pure human lung mast cell preparations, suggesting that cell types other than mast cells alone are involved in its generation (30).

Presently the only acceptable assay for SRS-A employs the anti-histamine-treated guinea pig ileum, but it is not specific for this mediator. Therefore, SRS-A must be further identified by its chromatographic properties, its susceptibility to inactivation by arylsulfatases, and its decreased assayability in the presence of a semispecific blocker, FPL55712.

Products of Arachidonic Acid Oxidation. In the human, products of arachidonic acid metabolism constitute the vast majority of prostaglandins and related compounds. Arachidonic acid mobilized from cell membrane phospholipids by the action of phospholipase A₂ is then either converted to prostaglandins and thromboxanes via a cyclooxygenase-dependent pathway or converted by a lipoxygenase to 12-L-hydroxy-5,8,10,14 eicosatetraenoic acid (HETE) and related compounds (Fig. 3). Several of these products have been

described in vitro subsequent to noncytolytic activation of lung tissue and rat mast cells. Specifically, PGD_2 , PGI_2 , and HETE have been generated by isolated rat mast cells, whereas $\text{PGF}_{2\alpha}$ and PGE_2 are present after IgE-dependent activation of human lung tissue (31, 33). Guinea pig central airway smooth muscle is constricted by $\text{PGF}_{2\alpha}$, thromboxane A_2 , and both cyclic endoperoxides PGG_2 . In the anesthetized dog, $\text{PGF}_{2\alpha}$ and PGD_2 both constrict central and peripheral airways. Additional pulmonary effects of generated prostaglandins are likely as elevations in cAMP and cGMP may be consequent to their actions (21). While prostaglandins of the E and F series may be identified by radioimmunoassay, other arachidonic acid products must be isolated and measured by thin layer chromatography or gas chromatography-mass spectroscopy.

Platelet Activating Factors (PAF). PAF are immunologically generated subsequent to IgE-dependent processes in human lung. Although PAF are themselves neither bronchospastic nor vasoactive, they induce platelet release of serotonin. This monoamine contracts several smooth muscle preparations, but it is without apparent bronchospastic effect in the human. PAF may also induce platelet aggregation and lead to the reversible sequestration of platelets in the lung. The best chemically described PAF are apparently phospholipids, susceptible to inactivation by phospholipases (17). PAF from different sources are not identical, as shown by the inability of rabbit PAF from two sources to deactivate platelets to the biologic effects of each other and by the somewhat different phospholipase inactivation profiles of rabbit leukocyte and rat peritoneal PAF.

Chemotactic Mediators

Eosinophil Chemotactic Factor of Anaphylaxis (ECF-A). The first mast cell-associated chemotactic factor described, ECF-A, was identified in the anaphylactic supernatant of challenged guinea pig lung fragments and subsequently in the analogous human tissue and isolated mast cells. This factor is preferentially chemotactic for eosinophils. It also deactivates this cell type to further migration. A small peptide of about 400 daltons, ECF-A has also been identified preformed in human lung and isolated mast cells (30). This mediator from human lung has been structurally characterized as two acidic tetrapeptides with the amino acid sequence Val/Ala-Gly-Ser-Glu (11).

Intermediate Molecular Weight Eosinophil Chemotactic Peptides. In addition to low molecular weight ECF-A tetrapeptides, human lung contains chemotactic factors of m.w. 1200-2500 with specificity for eosinophil polymorphonuclear leukocytes (2). These factors are preformed, immunologically releasable, and capable of deactivating eosinophils (5).

High Molecular Weight Neutrophil Chemotactic Factors (HMW-NCF). HMW-NCF has been described in rat mast cells, human lung fragments, and the serum of

patients with cold urticaria (39) or asthma (1) following challenge with ice water or antigen inhalation, respectively. HMW-NCF has been characterized as a 750,000-dalton neutral protein which attracts and deactivates neutrophils *in vitro*. HMW-NCF appears to be a sensitive marker for mast cell activation as it is present in serum following challenge of allergic asthmatics with doses of antigen insufficient to induce bronchospasm.

Histamine. In addition to its smooth muscle directed actions, histamine is active in modulating the migration of inflammatory leukocytes. Thus, via an H_1 mechanism, histamine enhances random and directed migration of eosinophils and neutrophils and is a weak chemotactic factor for human eosinophils. By its H_2 actions histamine inhibits both random and directed migration of neutrophilic and eosinophilic polymorphonuclear leukocytes (7).

Lipid Chemotactic Factors. The lipooxygenase product of arachidonic acid, HETE, has an ECF-A-like spectrum of activity. Unlike ECF-A, however, it increases eosinophil random migration and is a less potent chemotactic deactivator than ECF-A (13). Equivalent effects on neutrophils have also been described for the cyclooxygenase product 12-L-hydroxy-5,8,10-heptadecatrienoic acid (HHT) (13). A possibly related factor is a nonpolar lipid with chemotactic activity mainly for neutrophils which has been described in anaphylactic diffusates of rat mast cell-rich peritoneal cell preparations.

Structural Proteoglycans

Heparin. The sulfated, metachromatic, mucopolysaccharide heparin has been identified in human lung and localized to the mast cells isolated from human lung tissue (26). Human lung heparin is a proteoglycan of approximately 60,000 m.w. comprised of a protein core to which are attached, by xylosyl-seryl linkages, glycosaminoglycan side chains of average m.w. 20,000. Human heparin interacts with human antithrombin III to accelerate anticoagulation. Heparin from other sources has been shown to inhibit generation of the convertase enzyme (C3bBb) responsible for amplification of cleavage of the third component of complement (C3) (41), to inhibit binding of the C1q fragment of complement to immune complexes (24), to inhibit action of the activated first component of complement (C1s) upon its substrates, the fourth (C4) and second (C2) components (24), and to prevent binding of C2 to C4b (23). In addition, heparin has been shown to bind to platelet factor IV and to liberate lipoprotein lipase.

Granule-Associated Enzymes

Chymase. Chymase has been isolated from the mast cell of the rat and has been identified histochemically in the human mast cell (14). This enzyme is minimally active as a protease while stored in the granule, prob-

ably due to masking of the active site by heparin, but once freed its specific activity is comparable to that of pancreatic α -chymotrypsin. Chymotrypsin inhibitors have been shown to inhibit some inflammatory consequences of IgE-dependent activation in rabbit skin, adding relevance to the mast cell location of this activity (18).

Lung Kallikrein of Anaphylaxis. A 400,000 m.w. preformed enzyme termed lung kallikrein is released subsequent to antigen challenge of IgE-sensitized human lung fragments (40). This activity can cleave kininogen to yield bradykinin and can activate Hageman factor. The released kinins may contract bronchial smooth muscle and increase vascular permeability. Kinins have also been noted to induce bronchospasm when inhaled by asthmatic humans and have been detected in the serum of a single patient sustaining an acute asthma attack. Activation of Hageman factor with the induction of the clotting and fibrinolytic cascades could also be reasonably expected to ensue upon release of lung kallikrein.

Arylsulfatase. Arylsulfatase has been identified in human lung mast cells (30). Arylsulfatase A and B enzymes can inactivate SRS-A and, furthermore, arylsulfatase A is immunologically released with the mast cell granule in the rat (25).

Other Enzymes. β -glucuronidase and N-acetyl- β -D-glucosaminidase (hexosaminidase) have been identified in human lung and in rat mast cells and both are released upon immunologic activation of the rat mast cell (34). These enzymes in concert with arylsulfatase, when obtained from non-mast cell sources, have been demonstrated to degrade ground substance mucopolysaccharides.

MEDIATOR INTERACTIONS

Although the mediators of immediate type hypersensitivity have been identified and assessed for their effects as isolated factors, some understanding of their interactions is available. Thus the spasmogenic effects of histamine and SRS-A are synergistic and, in addition, the presence of either directly induces prostaglandin generation. The interactions of ECF-A, histamine, and ECF-oligopeptides are not fully delineated, but it is known that histamine in high concentrations inhibits and in low concentrations enhances ECF-A-induced eosinophil chemotaxis. In addition, the N and C terminal tripeptides of ECF-A are reversible and irreversible inhibitors of eosinophil chemotaxis, respectively (12). In addition, PGE_1 enhances and $PGF_{2\alpha}$ inhibits neutrophil chemotaxis, probably by their actions upon cyclic nucleotide concentrations. However, given the number and complexity of mast cell-dependent mediators other, potentially critical, biologic interactions await elucidation.

DETERMINANTS OF ACTIVITY OF MAST CELL MEDIATORS

Following activation of mast cells by IgE-dependent or other mechanisms, the panoply of mediators generated and released and the myriad of their potential interactions provide the substrate for induction of inflammatory events. The putative role for the mast cell and its mediators in both the homeostatic as well as pathophysiologic induction of inflammation in the lung is strengthened by the location of the mast cell at the respiratory surface. Thus the interaction of surface and intraluminal mast cells with antigen or noxious agents could, by the action of locally released permeability factors, lead to alterations of the respiratory epithelial barrier, thereby permitting access to the large number of deeper situated mast cells. Of more direct relevance has been the in vivo demonstration that solely mast cell-dependent events can indeed cause both immediate and more persistent inflammatory processes. The biphasic response of airways to inhaled antigen may be comparable to the biphasic cutaneous response to IgE-dependent mast cell activation (8, 36). In the cutaneous model a wheal-and-flare response reflects altered vascular permeability at the site, whereas later inflammatory events are accompanied by an intense cellular infiltration (36). Immunochemical studies have not revealed the participation of immune complexes or complement during this later inflammatory phase (36). Elicitation of both phases by purely IgE-dependent mechanisms indicates that mast cell activation can contribute to subacute and chronic as well as to acute pathobiologic processes. This fact is central to according the mast cell an important role in asthma, since this disease is characterized by acute exacerbations superimposed on chronic hyperirritability of the airway. Whether continued alteration in the threshold of airway response is due to mast cell mediators directly or to cellular infiltration is not established, but mast cell-derived mediators could be critical by either route alone or in combination.

The mechanism by which mast cell mediators might provoke such biphasic inflammatory responses is depicted in Figure 4. The mast cell-dependent generation of bronchospastic and vasoactive mediators establishes a local vasodilatory or humoral phase of inflammation while the release of chemotactic mediators provokes a cellular phase of inflammation. The humoral phase, apparent within minutes, would lead to egress from the circulation of immunoglobulin and complement as well as fibrinolytic, procoagulant, and kinin-generating proteins. This response could be expected to be rapidly beneficial to the host by aiding removal of invading microorganisms, by localizing and removing noxious agents, and by facilitating leukocyte migration through venular disconnections. On the other hand, an unregulated humoral phase might be expressed in disease as acute exacerbations of bronchospasm, alterations in tracheo-bronchial mucus flow or consistency, or as upper air-

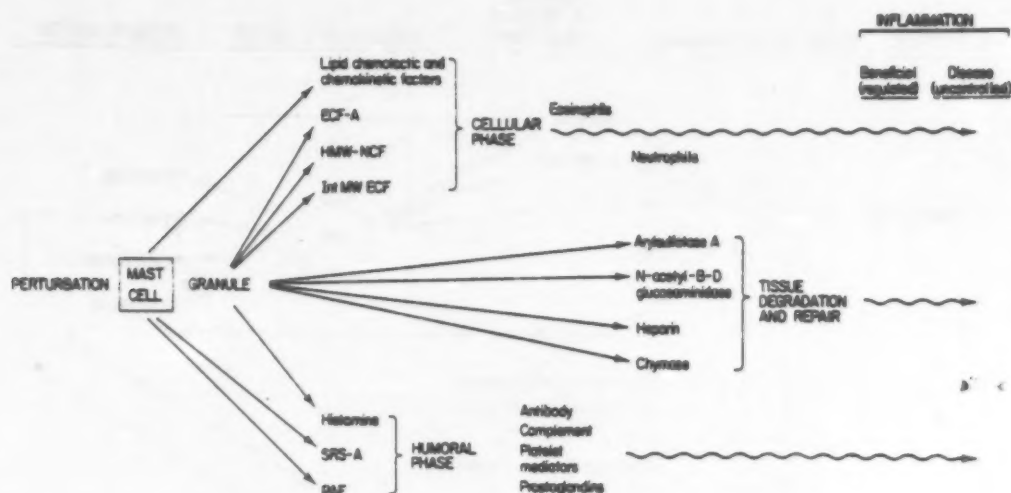


Figure 4. Schematic representation of mast cell-dependent humoral and cellular inflammatory events. [Modified from Austen: Triangle 17:109, 1978]

way obstruction due to edema. The generation and release of chemotactic factors would, in a period of several hours, be expected to call to the local inflammatory focus both eosinophilic and neutrophilic polymorphonuclear leukocytes. These cells could prove beneficial not only by their phagocytic function but also by their ability to inactivate mediators (see below). Conversely, if accumulation of leukocytes were uncontrolled, the pathobiologic consequences of tissue infiltration with leukocytes would ensue. Clinically, such infiltration might be seen in the inflammatory bronchial epithelium of asthmatic patients, in vasculitis, or perhaps, via the action of leukocytic lysosomal enzymes, in the destruction of lung tissue and the induction of fibrosis. By their capacities to deactivate leukocytes to further chemotactic activation, mast cell chemotactic mediators might also inhibit localization of leukocytes. In cold urticaria, following mediator release by experimental challenge, circulating neutrophils are rendered unresponsive to in vitro chemotactic stimulation. This effect is time limited but persists beyond the period in which measurable quantities of mediators are noted (6). Such an inhibitory action of chemotactic factors could prove beneficial by blunting exuberant inflammation or, if prolonged or ill-timed, might be harmful by preventing adequate host response to local insult. The release of mast cell proteases and lysosomal enzymes may themselves lead to alterations in ground substances and to activation of such protein inflammatory cascades as complement, fibrinolysis, and coagulation. These processes would amplify inflammatory events and might prove beneficial to the elimi-

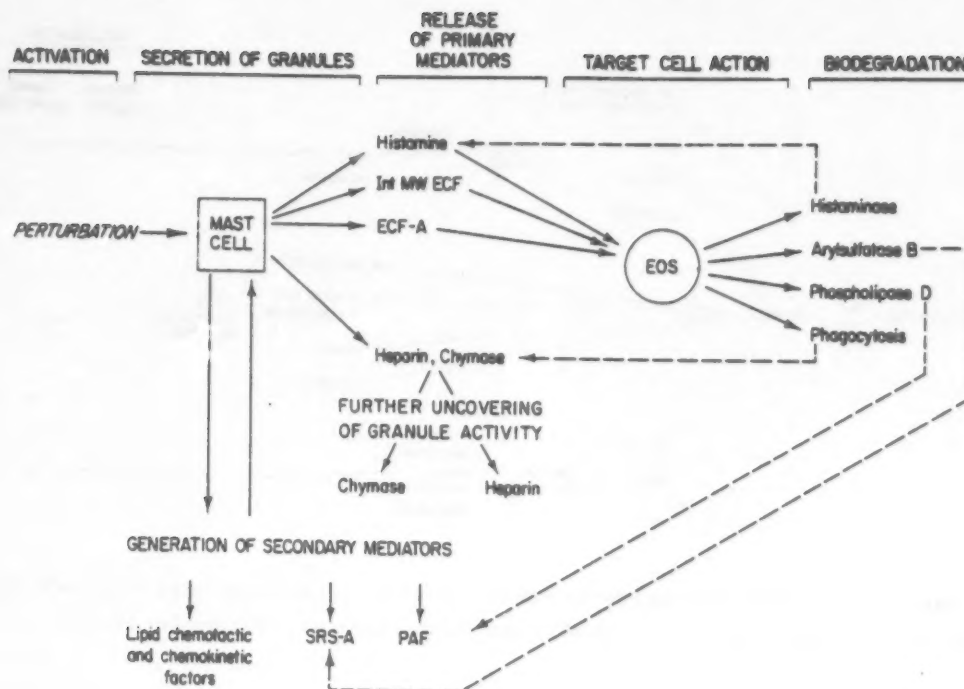


Figure 5. Schematic representation of endogenous regulation of mediator expression with particular emphasis on the role of the eosinophil. [Modified from Austen: Triangle 17:109, 1978]

nation of microorganisms or toxic agents but might also, if unregulated, lead to tissue destruction, chronic inflammation, and fibrosis.

REGULATION OF MAST CELL MEDIATOR RELEASE AND ACTIVITY

The events which lead to the generation and release of mast cell mediators and thereby to the elicitation of mast cell-dependent inflammatory events are subject to regulation at several critical points. Thus, activation of the mast cell, the secretion of granules, the release of the preformed mediators and generation of unstored mediators, the effect of mediators upon target cells, and finally the persistence in tissue of the mediators are all under biologic control (Fig. 5).

The extent and effect of activation of the mast cell are controlled by regulation of access of the eliciting agent to this cell, the amount of specific IgE antibody bound to the mast cell surface, and by the intensity of the stimulus. The mast cell itself might alter local permeability allowing increased antigen contact or, by the H_2 action of histamine, may suppress lymphocyte recognition of antigen and thereby prevent immune response to

invaders. In addition, the ratio of specific mediator generated is dependent on the intensity of the activating stimulus as limited stimulation of the mast cell leads to intracellular accumulation of SRS-A without release of SRS-A or histamine (22). Intracellular mast cell arylsulfatases may also control mediator generation by degrading SRS-A prior to its release.

Subsequent to activation of the mast cell the secretion of granules is under control of cyclic nucleotides as noted above. Of direct relevance is the possibility that the mast cell itself, by the mediator histamine via an H_2 action, and prostaglandins E_2 , D_2 , and I_2 may directly elevate cAMP and thereby inhibit granule secretion. Conversely, the H_1 action of histamine to elevate cGMP and that of $PGF_{2\alpha}$ to lower cAMP levels might augment mediator release.

Following secretion of the mast cell granule, the release of preformed mediators and thus the full expression of their activity is dependent on the independent solubilities of each of the mediators. Thus β -glucuronidase, arylsulfatase A, histamine, ECF-A and ECF-oligopeptides are fully soluble in physiologic buffers, hexosaminidase requires 0.5 M NaCl for full elution from the granule, and chymase-heparin complex requires 1.0 M NaCl for dissolution (19, 25). The relevance of granule binding is emphasized by the marked increase in proteolytic activity of the chymase following its elution from the granule. Although the mechanism(s) by which the mast cell granule is solubilized *in vivo* remains unknown, it is clearly an important step in regulating the activity of mast cell inflammatory mediators.

Regulation of the action of mast cell mediators upon target cells may derive from alterations in cyclic nucleotide concentrations, as noted above, or by the interaction of several mediators or their metabolites with the same target cell. Thus, histamine may be additive or inhibitory to ECF-A action upon eosinophils depending on the ratio of the two activities present. In addition, the constituent tripeptides of ECF-A are inhibitory to induction to eosinophil chemotaxis by ECF-A. As noted above, mediators may also be synergistic on smooth muscle as exemplified by SRS-A and histamine action.

The regulation of generation of unstored mediators has not been fully elucidated, but data suggest that the local microenvironment is critical to their generation. For example, SRS-A generation is maximal in mixed rather than in pure mast cell populations (30), and SRS-A, PAF, and lipid chemotactic factors can all be generated from mononuclear leukocyte populations. The ratios of critical target cells thus may be crucial to the amount and type of mediator generated.

Finally, the persistence of the effect of mediators is also regulated. While this may reflect the limited availability of key mediators, probable tachyphylaxis to some, and mediator clearance by excretion, it is also affected by enzymatic inactivation of these biologic activities. As many mast

cell-derived chemotactic mediators are eosinophilotactic, the eosinophil content of mediator-inactivating enzymes may provide a feedback control of mediator effects (Fig. 5). Notably, the eosinophil contains histaminase, arylsulfatase B, and phospholipase D, which inactivate histamine, SRS-A, and PAF, respectively. SRS-A is inactivated not only by extracted eosinophil arylsulfatase, but also by the enzyme-rich resting granulocyte, presumably after cellular uptake of the mediator. Some other cells also contain mediator-inactivating enzymes. Neutrophils contain histaminase, and at least one mononuclear cell population is rich in histamine methyltransferase. Interestingly, both mast cells and basophils contain arylsulfatase.

Inactivation of arachidonic acid metabolites is complicated, as some intermediate metabolic products are biologically active. Thus PGD_2 and thromboxane A_2 derived from the cyclic endoperoxides are potent bronchoconstrictors. However, most stable catabolic end products of arachidonic acid metabolism, such as thromboxane B_2 (from thromboxane A_2) and $\text{PG-6-keto-F}_{1\alpha}$ (from PGI_2), have little or no defined biologic activity.

CONCLUSION

The mast cell-derived preformed and newly generated mediators are released by IgE-dependent and independent mechanisms, and are biologically available during inflammatory events. The relevance of these mediators to allergic disease has been derived from studies of the pathophysiologic alterations induced by the individual mediators, identification of the mediators in tissue or biologic fluids of patients experiencing allergic reactions, and the known pathophysiology of the various atopic diseases.

Mediators of immediate hypersensitivity not only possess the ability to induce immediate tissue responses such as a wheal and flare, anaphylaxis, or rapid-onset brief-duration alterations in pulmonary function, but may also mediate a prolonged inflammatory response. The fact that IgE and mast cells are relevant to prolonged inflammatory events has been documented by passive transfer with isolated IgE of delayed inflammatory responses in skin and by the dependence on IgE antibody for similar delayed alterations in pulmonary mechanics following inhalation of antigen. In lung, these delayed responses are prevented by pretreatment with disodium cromoglycate, which supports the central role of the mast cell. Histopathologic assessment of delayed responses reveals an influx of neutrophils, eosinophils, basophils, lymphocytes, and mononuclear leukocytes, the deposition of fibrin, and vascular abnormalities which may progress to frank vasculitis (36). Although some of the mediators responsible for the early and later phases of the IgE-mast cell reaction can be surmised from the kinetics of their *in vitro* effects, their absolute identification and participation in disease require further definition.

The postulated role of the mast cell and its mediators in inflammation may provide insight not only into the clinical evolution of some allergic disorders such as progression of seasonal to perennial asthma, but also into the local homeostatic regulation of the lung environment and thereby the defense of the lung. Although much remains to be clarified, the rapidly expanding understanding of target cell activation together with the identification of mast cell-derived mediators provides a framework for definition of the complex processes that provide pulmonary defenses.

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DISCUSSION

PARTICIPANT: Did I understand you to say that the rat mast cell makes PGI_2 ?

DR. WASSERMAN: Yes, it does, with calcium ionophore. That is presented in abstract form, and was published in Federation Proceedings, 1978, by Lewis in collaboration with Roberts, Austen, and Oates.

PARTICIPANT: To tie this a little bit to air pollution in particular, as you mentioned at the end, the mast cells are sitting near the entry port. The paper of H. B. Thomas, in the late 1960s, showed that 0.5 parts per million nitrogen dioxide cause degranulation of submucosal mast cells in the rat lung. I think it would be interesting to ask why that did occur; after all, the mast cells are submucosal. How does the effect get down to the submucosa? Perhaps, there is some sort of opening of tight junctions that would allow an autonomic mediated response, or perhaps, antibody type material could even migrate down there.

DR. WASSERMAN: Mast cells are not solely submucosal. One of the more important recent observations is that they are both intraluminal and epithelial, and their availability to noxious stimuli, antigens, and so forth is obviously facilitated. It is presumed that their activation leads to increased access of materials to deeper structures by the action of vasoactive materials.

DR. GOLDSTEIN: On your slide you mentioned a role for lysophospholipids in degranulation, or in the membrane processes that occur. Is that something that's hypothesized?

DR. WASSERMAN: Yes. Both diacylglycerol and lysophotidylcholine have been postulated to enhance degranulation.

DR. GOLDSTEIN: Do you know it's an active compound, or do you have evidence for it?

DR. WASSERMAN: Lysophospholipids are known to be fusigenic in other systems. They cause lipid vesicles and platelets to fuse and they are very, very active materials. However, in recent work, Sullivan has not been able to find

lysophospholipids and has instead noted diacylglycerol, a product of phospholipase C, in mast cells. This material accumulates within three seconds in the mast cell, and it is also a very potent fusigen. It is possible that other fusigens are available. It's also conceivable that lysophospholipids are so active that when they're generated in mast cells they are rapidly degraded and thus are difficult to detect.

DR. BOUCHER: With respect to one of your last comments showing that mast cells are close to the lumen at least below the junctions, a lot of eosinophils seem to be present, proportionately, with them. Is there any data that suggest that these mast cells are, in fact, activated at that site?

DR. WASSERMAN: The association of the eosinophils and mast cells has been known for a long time. Somebody with a lot more time and patience than I have once ground up a cow, and in every tissue noted mast cells and eosinophils to occur together and generally at portals of entry. They are found throughout the upper and lower respiratory tract, gastrointestinal tract, urinary tract, and skin, although the eosinophils are not as prevalent in the skin. It's my prejudice that the eosinophil-mast cell axis that we described here is probably much more effective as a homeostatic regulator of low-grade chronic mast cell activation (which obviously must be ongoing at all times) than as an anaphylactic control. In fact, in many allergic reactions, the eosinophilic response is a marker for continued activation of the mast cells and for continued disease activity. It would be my prejudice that the eosinophils are noted near mast cells because of previous mast cell activation and the generation and release of specific eosinophil chemotactic factors.

Angiotensin Converting Enzyme:

I. New Strategies for Assay

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Angiotensin converting enzyme appears to occur in association with endothelial cells of virtually all vascular beds (for review see 22). The enzyme also occurs in association with brush border of renal proximal tubule (2, 30) and small intestine (31). However, bulk conversion of angiotensin I into angiotensin II occurs, apparently unaided by blood enzymes, within the pulmonary vascular space. Angiotensin converting enzyme is situated on the luminal surface of pulmonary endothelial cells (19, 23, 26) and is strategically placed for bulk processing, not only of angiotensin I but also bradykinin and perhaps other oligopeptides (neurotensin, enkephalins) having adequate affinity for the enzyme. The lungs have an enormous vascular bed through which flows the entire cardiac output. Further, because of the peculiar situation of the lungs within the general circulation, their venous effluent empties directly into the arterial circulation. Hence, the activity or inactivity of lung angiotensin converting enzyme may matter greatly to the quality of arterial blood and to the function of distant organs. Whether angiotensin converting enzyme is important to intrinsic lung function remains to be determined.

A major barrier to improved understanding of the pathophysiologic role of lung angiotensin converting enzyme is that of adequate techniques for assay. In principle, one should be able to measure modulations of enzyme activity by examining for variations in the pulmonary arteriovenous gradients of angiotensin I, angiotensin II, and bradykinin. However, these substances occur in blood at concentrations of less than 10^{-10} M. Radioimmunoassays can be used, but the precision of such assays is not great and their specificities,

especially for bradykinin, are not proved. The assay problem is not substantially improved by following the changes of activity of intravenously administered bradykinin or angiotensin I, either by radioimmunoassay or the dynamic, on-line bioassay system of Vane (29). Furthermore, the bioassay is not readily adaptable for use with human subjects.

In an effort to develop simpler and more precise means of measuring angiotensin converting enzyme, we have synthesized a series of acylated tripeptides, each bearing a radioisotope in the moiety used for acylation. Most have been prepared to contain a ^3H -atom, but gamma-emitting isotopes, e.g., ^{125}I , can be used as well. Angiotensin converting enzyme functions as a dipeptidyl carboxypeptidase and acts on acylated tripeptides to yield a dipeptide and an acyl-amino acid (for reviews see 1, 27). By careful selection of amino acid residues one can vary the affinity of substrate for enzyme. As has been shown for benzoyl-Gly-His-Leu (hippuryl-His-Leu; references 4, 5), the acyl-amino acid product can be separated from the acyl-tripeptide substrate by partitioning between acid-aqueous solvent and an organic solvent such as ethyl acetate or toluene. The efficiency of the extraction technique is a function of amino acid residues and their sequence. Thus an aromatic or aliphatic amino acid is advantageous for the ^3H -labeled product and a basic amino acid is useful when placed in the dipeptide leaving group.

Six radiolabeled acylated tripeptides have been prepared, and four have been characterized in detail as substrates for angiotensin converting enzyme. Three of the latter compounds can be used to measure converting enzyme of lungs perfused with artificial salt solutions. We believe that these three substrates can be used to measure lung converting enzyme in vivo.

MATERIALS AND METHODS

Synthetic bradykinin and its higher homologs, angiotensin I and its lower homologs, and BPP_{9a} and its lower homologs were prepared by the solid phase peptide synthesis technique (28). The completed peptides were purified by two or more of the following techniques: counter current distribution, molecular sieve chromatography (Bio-Gel P-2, Sephadex G-25), and partition chromatography (LH-20 within 6% butanol in H_2O ; Sephadex G-25 with butanol, acetic acid, H_2O ; 4:1:5). The peptides were synthesized by J. M. Stewart, University of Colorado Medical Center, and G. H. Fisher, University of Miami (12).

The compounds, p-I-benzoyl-Gly-Gly-Gly, p-I-benzoyl-Pro-Phe-Arg, p-I-benzoyl-Gly-His-Leu, p-I-benzoyl-Phe-Ala-Pro, p-I-benzoyl-Phe-His-Leu, and p-I-benzoyl-Phe-Ser-Pro, were synthesized by step-wise solution methods (17, 18). Purifications were accomplished with one or more of the following

techniques: molecular sieve chromatography (Sephadex G-10), ion-exchange chromatography (CM-Sephadex or DEAE-Sephadex with ammonium acetate buffers). The p-³H-benzoyl-derivatives were prepared by New England Nuclear Corp. by dehalogenation in ³H₂ gas at 39 lb./in.² for 1 hour over 5% rhodium on calcium carbonate in dimethyl-formamide and H₂O, 1:1 (vol:vol). Each of the ³H-labeled acyl-tripeptides was obtained at specific radioactivities of >20 Ci/mmol. The ³H-labeled compounds were purified on Sephadex G-10, and each behaved as a pure substance in two or more thin layer chromatography systems (18) and on paper electrophoresis at pH 2.0 and 5.0 (20). The ³H-labeled compounds, stored at 1 mCi/ml of ethanol at -28°C, did not undergo detectable radiolysis over a period of 12 months.

Our studies used angiotensin converting enzyme partially purified from guinea pig lung or urine and human urine (18). Pure rabbit lung angiotensin converting enzyme was provided by R. L. Soffer, Cornell University Medical College (27).

The experiments using anesthetized rats or perfused isolated rat lung were performed as described by Roblero *et al.* (16) and Ryan *et al.* (24). For *in vitro* assays, we used the protocol described by Ryan *et al.* (17). In brief, reactions were stopped by adding 0.1N HCl to a pH <2. The ³H-labeled acyl-amino acid product was extracted into an equal volume of organic solvent. Ethyl acetate was used for all substrates except ³H-benzoyl-Phe-Ala-Pro. Toluene was used for extraction of reaction mixtures containing the latter substrate (18).

RESULTS AND DISCUSSION

Recognition Sites of Angiotensin Converting Enzyme

In the early 1970s, it was recognized that angiotensin converting enzyme is misnamed. The enzyme acts as a dipeptidyl carboxypeptidase capable of removing the C-terminal dipeptide of a variety of oligopeptides (1, 9, 11). In fact, bradykinin is a better substrate (lower K_m) than angiotensin I, and the enzyme is sometimes known as kininase II. In terms of K_m , the venom peptide, BPP_{5a} (13), is the best of the known substrates ($K_m < 10^{-6}$ M).

Clearly, the enzyme is not specific, but there is reason to believe that the enzyme is highly selective. Further, it is evident that the enzyme possesses a number of recognition sites, knowledge of which is useful for design of synthetic substrates.

In our early studies on the metabolism of kinins by intact lungs, we noted that bradykinin is inactivated more rapidly than any of its N-extended higher homologs (16, 21). When pure lung angiotensin converting enzyme became available (8), it was found that much of the selectivity of processing

of kinins by intact lungs could be explained in terms of one enzyme (see Table 1). Clearly, the enzyme is sensitive to amino acid residues at a distance from the cleavage site. Chain length and charge of the substrates appear to be important factors.

Angiotensin I and its lower homologs are also treated differently by converting enzyme. As shown in Table 2, des-Asp¹-angiotensin I is a somewhat better substrate than angiotensin I itself (lower K_m , higher V_{max}/K_m). In terms of V_{max}/K_m , the C-terminal pentapeptide is a better substrate than angiotensin I. In comparison with some of the acylated tripeptide substrates described below, the C-terminal hexapeptide is a surprisingly poor substrate. Nonetheless, Table 2 emphasizes the points that converting enzyme is sensitive to amino acid residues distant from the cleavage site and that chain length is an important factor.

Greater insight into the importance of enzyme recognition sites has come from studies of two inhibitors of converting enzyme, BPP_{9a} (<Glu-Trp-Pro-Arg-Pro-Gln-Ile-Pro-Pro) and BPP_{5a} (<Glu-Lys-Trp-Ala-Pro), peptides purified from the venom of *Bothrops jararaca* (13, 15). Cushman *et al.* (7) have examined for the inhibitory potencies of a series of analogs of the two inhibitors, and we (12) have examined for the inhibitory potencies of BPP_{9a} and its lower homologs.

Table 1. Processing of Bradykinin and Its Higher Homologs by Isolated, Perfused Rat Lungs and by Angiotensin Converting Enzyme Purified from Pig Lung

	Intact lungs (%)	Converting enzyme (%)
Bradykinin (BK)	100	100
Lys-BK	96	65
Met-Lys-BK	80	55
Lys-Lys-BK	0	12
Gly-Arg-Met-Lys-BK	<10	5
Polistes kinin	0	2

See reference 11 for further details. Results are expressed as relative reaction rates (the rate for bradykinin = 100%). Rates of hydrolysis by pure enzyme were measured in terms of the formation of ninhydrin-reactive products (9). Rates of degradation of kinins by isolated lungs were measured by bioassay (16, 21).

Table 2. Reaction of Pig Lung Angiotensin Converting Enzyme with Angiotensin I and Its C-terminal Lower Homologs

	K_m (μM)	V_{max}	V_{max}/K_m
Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu	33	1.98	0.060
Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu	11	0.99	0.090
Val-Tyr-Ile-His-Pro-Phe-His-Leu	170	7.94	0.047
Tyr-Ile-His-Pro-Phe-His-Leu	100	8.83	0.088
Ile-His-Pro-Phe-His-Leu	1,000	37.71	0.038

For experimental details see reference 3. Reaction rates were measured in terms of the appearance of the fluorophor formed by reacting His-Leu with o-phthaldialdehyde. V_{max} is expressed in terms of $\mu mol/min/mg$ of enzyme protein.

Both studies have shown that Trp of BPP_{9a} interacts with an important recognition site, apparently distant from the catalytic site of the enzyme. However, the distant binding site may not be fixed or stationary, as the N-terminal homolog of BPP_{9a}, <Glu-Trp-Pro-Arg-Pro-Gln-Ile, and its lower homolog, Trp-Pro-Arg-Pro-Gln-Ile, are virtually as potent as is BPP₉ itself (respective I_{50} values: 54, 34 and 28 nM). Results obtained with some of the homologs are shown in Table 3.

At present, it appears that binding of a substrate or inhibitor to angiotensin converting enzyme is initiated by interaction of the free α -carboxy group of the substrate with an ionic recognition site. Our results and those of others indicate that the ring of proline or the side chain of isoleucine aids in binding (6, 14). Clearly, the side chains of C-terminal arginine, as in bradykinin, and leucine, as in angiotensin I, are not unfavorable for binding. Similarly, phenylalanine and histidine are not unfavorable penultimate residues, but alanine (as in BPP_{5a}) may be the most favorable. Less attention has been paid so far to the importance of the side chain of the third amino acid residue; i.e., that on the N-terminal side of the cleavage site. Such data as are available indicate that an aromatic amino acid (e.g., Trp of BPP_{5a} or Phe of angiotensin I) is favorable for binding. Judging from the low I_{50} of the C-terminal tripeptide of BPP_{9a}, Ile-Pro-Pro (Table 3), the side chain of isoleucine fits well. Through such considerations, we have prepared six acylated tripeptide substrates for converting enzyme.

Table 3. Inhibition of Angiotensin Converting Enzyme by BPP_{9a}
and Some of Its Lower Homologs (12)

Structure	I ₅₀ (nM)
<Glu-Trp-Pro-Arg-Pro-Gln-Ile-Pro-Pro	28
Trp-Pro-Arg-Pro-Gln-Ile	34
Trp-Pro-Arg-Pro-Gln-Ile-Pro-Pro	50
<Glu-Trp-Pro-Arg-Pro-Gln-Ile	54
Pro-Arg-Pro-Gln-Ile-Pro	170
Ile-Pro-Pro	330
Gln-Ile-Pro-Pro	760
Arg-Pro-Gln-Ile-Pro-Pro	1,100
Pro-Gln-Ile-Pro-Pro	1,500
<Glu-Trp-Pro	42,000
<Glu-Trp-Pro-Arg	450,000

Compounds were tested for their ability to inhibit the hydrolysis of [³H]hippuryl-His-Leu by human urinary angiotensin converting enzyme (18).

Design of Synthetic Substrates

We have synthesized the following compounds: p-I-benzoyl-Gly-Gly-Gly (I), p-I-benzoyl-Pro-Phe-Arg (II), p-I-benzoyl-Gly-His-Leu (III), p-I-benzoyl-Phe-Ala-Pro (IV), p-I-benzoyl-Phe-His-Leu (V), and p-I-benzoyl-Phe-Ser-Pro (VI). Each of the compounds can be labeled by dehalogenation in ³H₂ gas (17, 18). Compounds I-V have been so labeled and each was obtained at a specific radioactivity of >20 Ci/mmole. Further, each compound was labeled specifically in position 4 of the benzoyl ring.

Compound I, after tritiation, is [³H]hippuryl-Gly-Gly. Hip-Gly-Gly is commonly used as a substrate for converting enzyme (10) and appears to be remarkably resistant to hydrolysis by other enzymes. However, (shown in Table 4), Hip-Gly-Gly has relatively little affinity for converting enzyme, and its ³H-derivative cannot be used to obtain a sensitive assay.

Compound II, the acylated C-terminal tripeptide of bradykinin, yields a much more sensitive assay, although its V_{max}/K_m suggests that one or both of the products is a poor leaving group. Under some conditions, II is hydrolyzed by a carboxypeptidase B-like enzyme and is not specific. The encouraging results obtained with III, Hip-His-Leu, deserve comment, as the magnitude of its K_m is heavily dependent on selection of buffer and salts. Cushman and

Table 4. Characteristics of the Reactions of
Acylated Tripeptides with Angiotensin Converting Enzyme (18)

Substrate	$V_{\max}$ ($\mu\text{M}/\text{min}$)	K_m (μM)	$\frac{V_{\max}}{K_m}$
I. benzoyl-Gly-Gly-Gly	83.3	5000	0.0167
II. benzoyl-Pro-Phe-Arg	1.4	172	0.0081
III. benzoyl-Gly-His-Leu	5.6	177	0.0316
IV. benzoyl-Phe-Ala-Pro	0.091	12.5	0.0073

Each of the ^3H -labeled substrates, with varying concentrations of unlabeled carrier, was reacted with guinea pig urinary angiotensin converting enzyme such that no more than 10% of initial substrate was used. Similar results are obtained when rabbit lung converting enzyme is used.

Cheung (4, 5) were the first to use Hip-His-Leu as a substrate for converting enzyme, and under their conditions of assay (0.4M phosphate buffer, pH 8.3, plus 0.3M NaCl), Hip-His-Leu has a relatively low affinity for the enzyme ($K_m > 2000 \mu\text{M}$). Quite by accident, we found that its affinity is greatly increased by Na_2SO_4 at 0.75M (10). Phosphate is inhibitory; thus the most sensitive assay uses [^3H]Hip-His-Leu in 0.05M HEPES buffer, pH 8.0, plus 0.1M NaCl and 0.75M Na_2SO_4 . As yet, we have no explanation for the dramatic effects of SO_4 . Nonetheless, our work and that of Dorer *et al.* (10) indicate that it will be important to check for effects of organ perfusion solutions (e.g., Krebs-Henseleit solution) on the interactions of a given substrate with angiotensin converting enzyme. Plasma and blood are likely to present their own problems.

Compound IV, an acylated analog of the C-terminal tripeptide of BPP_{5a}, can be used in assays 400 times more sensitive than those using Hip-Gly-Gly (I). Neither II nor IV requires Cl^- , and each is apparently insensitive to added Na_2SO_4 . The K_m for IV is intermediate between that for angiotensin I (33 μM) and that for bradykinin (0.85 μM) (9, 27).

CONCLUDING REMARKS

Depending on the goals of the experiment, compound I, III, or IV can be used to measure angiotensin converting enzyme of plasma, tissue homogenates, cells in culture, or isolated, perfused lungs. Compound IV may well be suitable for use with lungs of intact animals. Because of the wide range of affinities of the substrates for converting enzyme and their high specific radioactivities,

it should be possible to examine for modulations of converting enzyme activity using a wide range of substrate concentrations. Each of the ^3H -labeled substrates, without carrier, is labeled such that 1 nM solutions contain easily detectable radioactivity. Thus the range of concentrations readily tested extends from 1 nM to greater than twice K_m . So far as is known, none of the substrates or products is biologically active or toxic. The acyl-tripeptides are relatively inexpensive and can be made in quantity.

Again, according to the goals of a given experiment, an acyl-tripeptide substrate can be selected to give <10% to >90% hydrolysis during one transit through the lungs. Thus, it should be possible to define conditions to assess easily effects of variables such as perfusion rate, pressure, toxic inhalants, and oxygenation.

ACKNOWLEDGMENTS

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Angiotensin Converting Enzyme: II. Pulmonary Endothelial Cells in Culture

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One of the technical limitations to progress in understanding specific functions of a particular cell type is the availability of that cell type in pure culture. Over the past 12 years, a number of different specific metabolic activities have been attributed to pulmonary endothelial cells. The cells can selectively process adenine nucleotides and biogenic amines (for review see 7 and 8). In addition, endothelial cells are capable of forming prostaglandins such as PGE_2 (19, 20, 24) and PGI_2 (15, 32). Endothelial cells appear to have a great capacity for inactivating kinins and for converting angiotensin I into angiotensin II (for review see 19 and 25) and, in this sense, endothelial cells of the pulmonary vascular bed can regulate the hormonal composition of systemic arterial blood. In addition, endothelial cells appear to possess a number of hemostatic factors including α_2 -macroglobulin (1), plasminogen activator (31), and factor VIII antigen (11).

By and large, metabolic activities attributed to pulmonary endothelium were based on conclusions drawn from indirect evidence. Perhaps the most direct method to examine for unique or characteristic properties of a given line of endothelial cells is to use isolated cells (17, 22, 30) or cells in culture. Others have developed methods for isolating and culturing endothelial cells from human umbilical vein (9, 12, 13, 16). Similar techniques have been used to isolate and culture endothelial cells from portal vein and aorta (2, 3, 14, 28, 29). Therefore, to improve our ability to examine directly for specific metabolic activities of pulmonary endothelium, we have begun a program to obtain pulmonary endothelial cells in culture. Two methods have

been developed: 1) cells can be obtained from pulmonary artery and vein of large animals (cow and pig) and 2) cells can be obtained from the microvasculature of small animals (rats, guinea pig, and rabbit) commonly used in studies of "nonventilatory functions" of the lungs. The latter technique can also be used to obtain cells from a lobe of lung from large animals and may be adaptable for use with human tissue obtained by open biopsy.

MATERIALS AND METHODS

Isolation and Culture of Pulmonary Artery Endothelial Cells

Pulmonary arteries from newborn or unweaned calf (veal) are obtained within 30 minutes of slaughter. Calf hearts with attached great vessels are transported at 4°C to the laboratory where they are rinsed with Puck's saline. Each pulmonary artery is separated from the aorta and trimmed of excess fat and connective tissue. The pulmonary artery is tied as close to the heart as possible and the vessel is removed. Each vessel is washed by immersion in three changes of Puck's saline containing 3x strength antibiotics (Biostat 100, 3 ml per liter). When all of the vessels have been trimmed and washed, they are placed in a beaker of Puck's saline with 3x antibiotics and stored at 4°C for about 90 minutes. This storage step appears to reduce the likelihood of subsequent bacterial contamination. The vessel segments are then warmed to 37°C in a shaking water bath. Each pulmonary artery segment is drained of excess saline and filled with a solution of 0.25% collagenase (Worthington type CLS II) in Puck's saline containing normal strength antibiotics (Biostat 100, 1 ml per liter). The free end of each artery is then clamped with a large hemostat. The arteries are suspended in a 2-liter beaker of phosphate buffered saline and incubated at 37°C in a shaking water bath for 25 minutes. The arteries are then removed and the exteriors blotted dry on sterile tissue. Each artery is then suspended by the ligature over a small beaker or centrifuge tube and the hemostat is released. The collagenase mixture containing cells is decanted into 15-ml tubes and centrifuged at $750 \times g$ for 10 minutes at 4°C. The supernatant is discarded and the pellets washed by resuspending and repelleting twice with cold medium 199 (without fetal calf serum). The final pellets are resuspended in 1 ml of medium 199 and seeded into 25-cm² tissue culture flasks containing 4 ml of medium 199 with 20% fetal calf serum and normal strength (1 ml per liter) Biostat 100. The flasks are capped loosely and placed in a CO₂ (5%) incubator at 37°C. The cells show the greatest plating efficiency when seeded as small clumps rather than as monodispersions. Hence, hemocytometer counts may not be accurate. However, cells seeded at densities of approximately $1.5-3 \times 10^5$

per flask reach confluency within five days. At confluence, the cells reach a density of about 4×10^4 per cm^2 (10^6 per flask). The medium is changed every two days.

The cells can be subcultured as follows: Flasks are washed for about 10 minutes with 5 ml of Ca^{++} and Mg^{++} -free Puck's saline at 37°C . The saline wash is aspirated off and the cells are incubated with 0.05% trypsin with 0.02% EDTA in Ca^{++} and Mg^{++} -free Puck's saline (5 ml at 37°C). The cells are monitored on an inverted microscope to determine the optimal length of time of exposure to the trypsin-EDTA solution. We have found that after 3 minutes the small, polygonal endothelial cells begin to lift off while the larger, flatter smooth muscle cells still adhere to the flask. With a minimum of agitation, the enzyme mixture containing cells is transferred, using a 5-ml pipet, to 15-ml conical centrifuge tubes. The cells are centrifuged at $750 \times g$ for 10 minutes. The trypsin solution is removed by aspiration and the pellet is resuspended in a small quantity of medium 199 without fetal calf serum. The flasks are usually split two for one. Each new flask is seeded with approximately 10^5 cells. The doubling time for bovine pulmonary artery endothelial cells is about 24 hours. Flasks which are predominantly endothelial (less than 5% contamination with smooth muscle cells) can be purified by successive subculture, taking advantage of the difference in attachment and detachment rates of the two cell types. Trypsin preferentially detaches bovine endothelial cells; thus, short periods (2 to 3 minutes) of exposure to trypsin prior to transferring cells tend to leave smooth muscle cells adherent to the flasks. Furthermore, endothelial cells are the first to reattach. Hence, cells are seeded into new flasks and incubated for one hour at 37°C . Medium and any free-floating cells are removed by aspiration and replaced with fresh medium containing fetal calf serum and antibiotics.

Isolation and Culture of Endothelial Cells from the Lungs of Small Animals

The techniques described above for the isolation and culture of endothelial cells from bovine pulmonary artery have provided useful information on specific metabolic activities of pulmonary endothelium (for review see 19). But the techniques are not suitable for obtaining endothelial cells from the vessels of small animals or from the microvasculature. We have, therefore, developed a technique to obtain endothelial cells from the lungs by vascular perfusion with collagenase in a HEPES buffered salt solution. The surgical procedure and techniques for collecting cells are similar using rats, rabbits, or guinea pigs of either sex (see Figure 1).

The following describes the perfusion system used with a female New Zealand white rabbit weighing 2.16 kilograms: A tracheostomy was performed and a

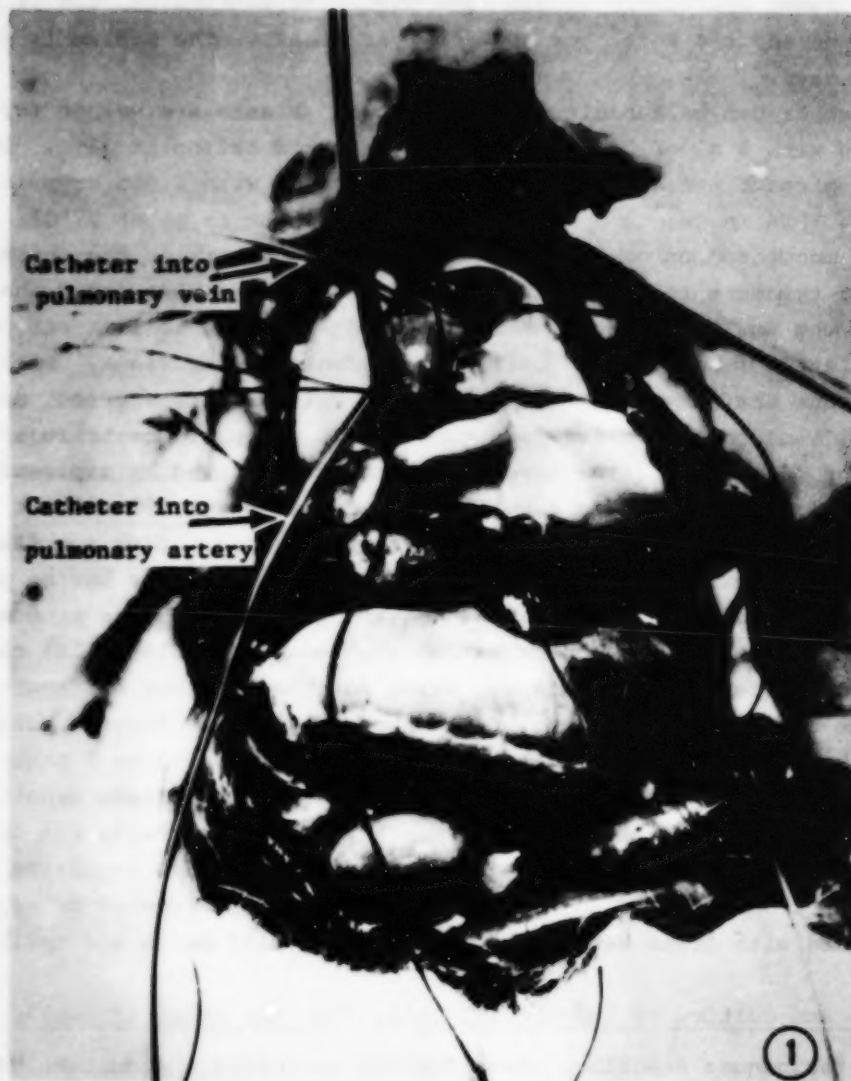


Figure 1. Perfusion system for (a) rendering lungs free of blood and (b) treating the microvasculature with collagenase to allow collection of endothelial cells. The perfusion system is described in the text. Small gauge catheters are inserted and tied into the pulmonary artery and pulmonary vein sinus. Saline is perfused in a forward direction (artery to vein) until the effluent is free of blood. Perfusion of enzyme is from the venous to the arterial side, a maneuver accomplished by connecting the drain cannula (in the pulmonary vein) to the enzyme stopcock and by disconnecting the cannula to the pulmonary artery from the saline stopcock. The free end of the arterial cannula is then directed to a collection tube.

tracheal tube was inserted, secured with a ligature, and attached to a Harvard respirator (tidal volume 18 ml, 45 strokes per minute, inspiration to expiration ratio of 0.45). Heparin (5,000 units) was injected intravenously. Ligatures were placed around the pulmonary artery and the left atrium to create a venous sinus into which all pulmonary veins drained. The saline perfusion system was opened to the arterial catheter. The pulmonary artery was opened and the catheter inserted and secured with a ligature. A hole was immediately cut in the right ventricle for drainage; a hole was also cut in the left ventricle and a drain catheter inserted through the mitral valve and into the atrium and secured with a ligature. The perfusion solution was pumped for 5 to 10 minutes, at which time the effluent was clear and colorless. When the lungs became white and their venous effluent was free of blood, the collagenase stopcock was opened, the respirator was turned off, and the trachea was clamped. The free end of the pulmonary vein catheter was attached to the collagenase stopcock. The pulmonary artery catheter was detached from the saline perfusion system and directed to a cell collection tube. Thus, retrograde perfusion of the lungs was established. The free end of the pulmonary artery catheter was directed into a sterile 15-ml centrifuge tube to collect the cell-enzyme suspension. Perfusion with collagenase was continued until the lungs became edematous and flow stopped spontaneously. The collection tubes were centrifuged for 10 minutes at $750 \times g$. The supernatant was removed by aspiration and each pellet was resuspended in 10 ml of 0.01 HEPES buffered saline, pH 7.4. The tubes were centrifuged again and the supernatant discarded. The pellets were resuspended in 1 ml of medium 199 using a Vortex mixer. Corning tissue culture flasks, 25 cm^2 , were filled with 4 ml of medium 199 containing 20% fetal calf serum and antibiotics. Each culture flask was seeded with 1 ml of suspension and the flasks were incubated at 37°C in 100% humidity in a 5% CO_2 atmosphere. The number of cells seeded per flask is approximately 2.8×10^5 . However, the seeding density cannot be determined accurately since the cells are removed as clumps or sheets, not as a monodispersion, and because blood cells are present (and beneficial) in the original isolate. Those flasks containing more than 95% endothelial cells (as judged by phase contrast microscopy) are retained for culture, and the lines are purified over the next few passages using differential adherence procedures as described above.

RESULTS AND DISCUSSION

Identification of Endothelial Cells

Cells obtained by either method are readily identifiable as endothelial according to morphological and functional criteria. When examined in the

inverted microscope by phase contrast microscopy, the cells grow as monolayers with a cobblestone appearance characteristic of endothelium (Figs. 2 and 3). When examined in the electron microscope, they contain all of the cellular organelles expected of pulmonary artery endothelial cells in situ, including Weibel-Palade bodies (Figs. 4 and 5). The cells are routinely examined by electron microscopy of thin sections of monolayers still attached to the culture flasks (23, 28). However, we have developed means of examining the cells in culture without removal from the culture flasks by additional techniques including freeze-fracture of monolayers, scanning electron microscopy, and examination of surface replicas (10). Thus, we are now able to recognize a variety of views of endothelial cells. In addition, our cultures possess functional aspects characteristic of endothelial cells. The cells possess converting enzyme activity as demonstrated by using [^{125} I]Tyr⁸-bradykinin as substrate (4, 26, 28) and a variety of synthetic substrates (see paper by Ryan et al. in this volume). In addition, the cells are reactive with antibodies to angiotensin converting enzyme as can be shown with immunofluorescence microscopy and immunocytochemistry (28). Using the latter technique, we were able to localize angiotensin converting enzyme on the plasma membrane and caveolae of endothelial cells in culture, a result in accord with our previous demonstration of angiotensin converting enzyme on the luminal surface of pulmonary endothelial cells in situ. Bovine endothelial cells also react with antibodies to human factor VIII and α_2 -macroglobulin, and this we have demonstrated by immunofluorescence (23).

Synthesis of Prostaglandins by Pulmonary Endothelial Cells

Results of our studies show that bovine pulmonary artery endothelial cells are capable of forming prostaglandins and related substances from 1- ^{14} C-arachidonate. Using EM autoradiography of cells incubated with [^{3} H]-acetyl salicylate (a specific inhibitor of prostaglandin endoperoxide synthase), we were able to show that the enzyme is situated on the endoplasmic reticulum (20, 24, 27). PGE₂ is the major metabolite. A substance which co-chromatographs with 6-keto-PGF_{1 α} , a breakdown product of PGI₂, is also formed, but in highly variable quantities. Synthesis of PGI₂ by endothelial cells has been recently described by a number of laboratories (e.g., see 32). It is likely that part or all of the PGI₂ formed by lungs arises from endothelial cells. Synthesis of PGI₂ by pulmonary endothelial cells, along with their ability to degrade ADP (see below), may account in large part for the capacity of endothelial cells to prevent or inhibit platelet aggregation. We were surprised to find that PGB₂ occurs as a major product. Conceivably, PGB₂ is formed from PGE₂ during extraction. If so, the rate of PGE₂ synthesis

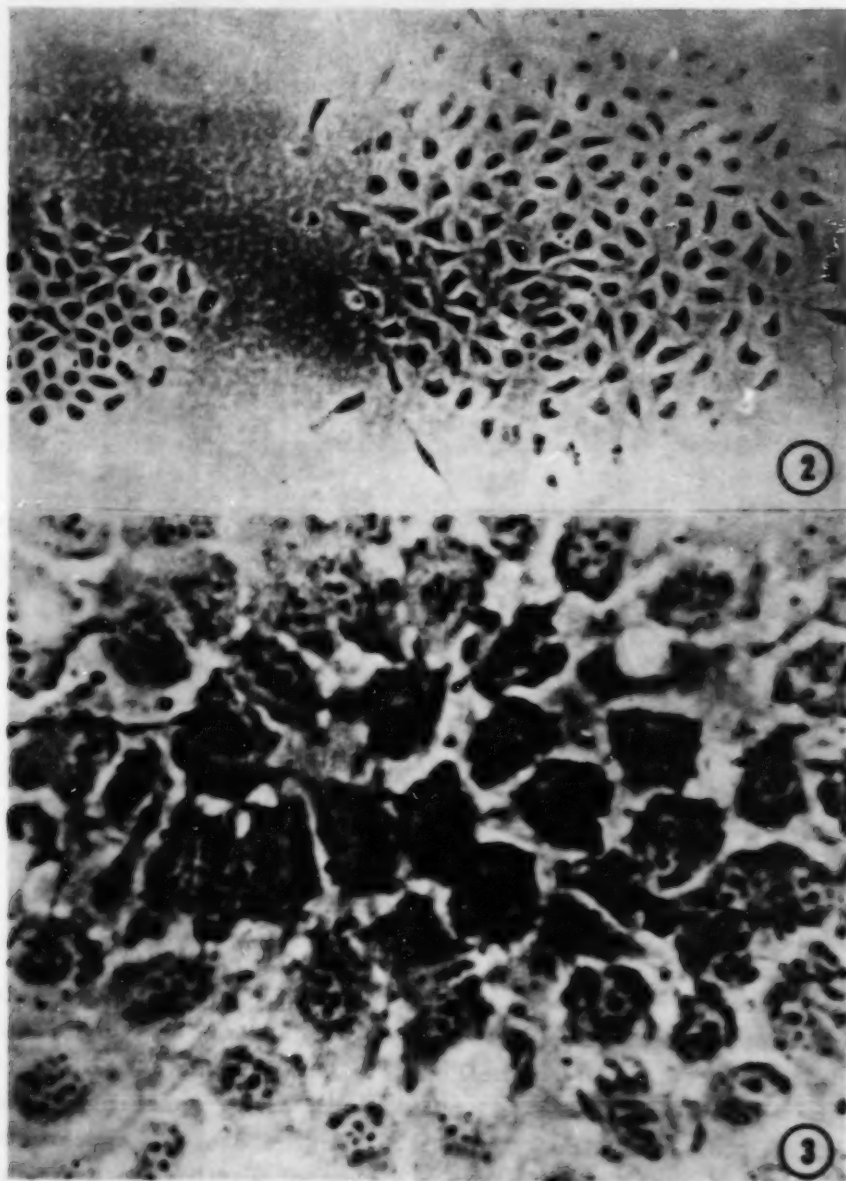


Figure 2. Phase contrast micrograph of cow pulmonary artery endothelial cells after 21 hours in culture. The cells form a cluster of approximately 100 cells per cluster. X150.

Figure 3. Confluent monolayer (approximately 4×10^4 cells/cm²) of cow pulmonary artery endothelial cells. Phase contrast. X750.

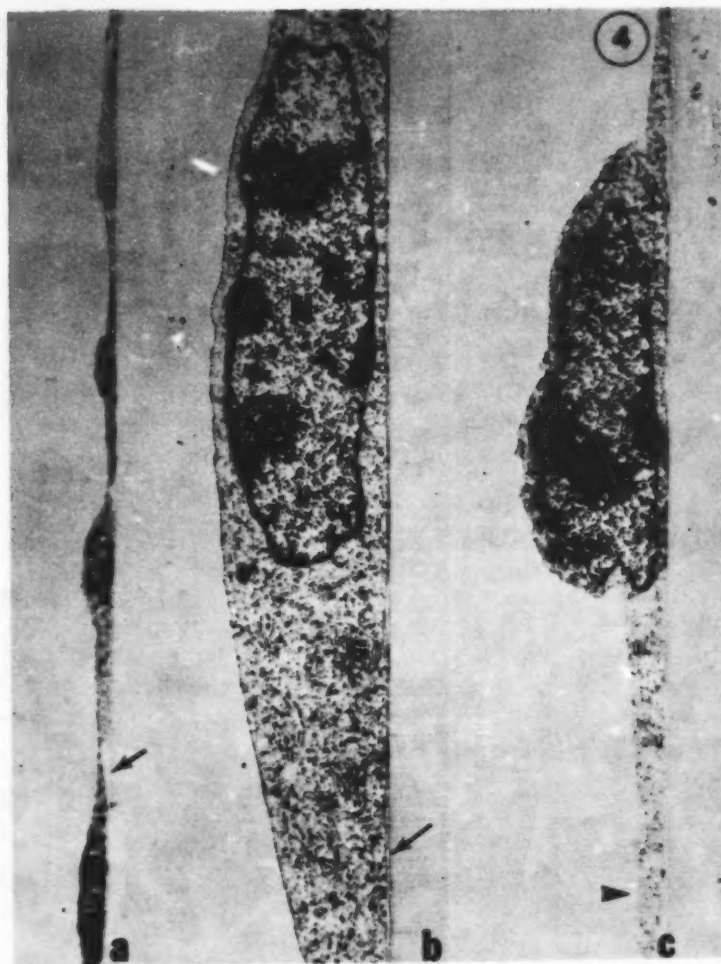


Figure 4. Electron micrographs of calf pulmonary artery endothelium sectioned transversely, including a portion of the plastic culture flask on which the cells were growing (F). The boundary between the flask and the cell is delineated by a fine dense line indicated by an arrow (probably formed from components of PCS) (see also Figure 5). (a) The overall appearance of a monolayer in transverse section and the contact inhibition between neighboring cells. X1,680. (b) The main body of the cell containing rough (*) and smooth ER. Golgi complexes (G) are frequently found around the nucleus (N). X8,800. (c) The thick portion of the cell in the region of the nucleus and the slender peripheral extensions. These extensions do not contain abundant endoplasmic reticulum but are characterized by numerous microtubules and microfilaments. Caveolae (arrowheads) occur on both the peripheral and central portions of the cells. X5,500. Reproduced with permission from *Tissue & Cell* 10:535-554, 1978.

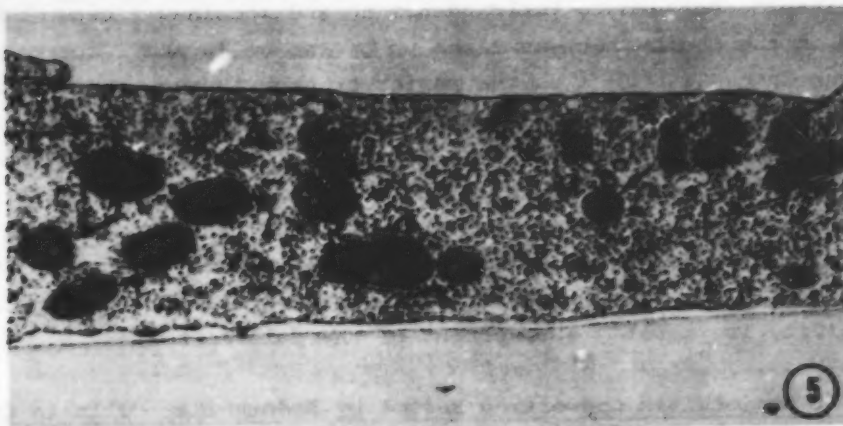


Figure 5. Thin section of endothelial cell showing Weibel-Palade bodies (arrows). X32,000.

approached 0.5 nm/ μ g of cell protein per hour. The possibility should be considered that pulmonary artery endothelial cells have an unusually large capacity for synthesizing PGE_2 , a vasodilator thought to prevent or lessen hypoxic pulmonary vasoconstriction. Our results indicate that pulmonary endothelial cells can account for part of the efflux of prostaglandin-like substances from embolized or anaphylactic lungs. However, our results provide no information on the cellular origins of thromboxane A_2 , likely to be a primary mediator of anaphylaxis, or of substances such as 15-keto-dehydro-metabolites of PGE_2 and $\text{PGF}_{2\alpha}$, all compounds which may occur in lung venous effluent in much higher concentrations than those of PGE_2 and $\text{PGF}_{2\alpha}$. Previously, we have shown that endothelial cells in culture cannot degrade $\text{PGF}_{1\alpha}$ and E_1 , excellent substrates for 15-OH-dehydrogenase (19).

Endothelial Cells and the Degradation of ADP

Intact blood vessel walls are known to have anti-thrombotic properties, thought to be associated with the endothelial cell lining layer. As mentioned above, it has recently been shown that endothelial cells in culture can synthesize prostacyclin (PGI_2), a substance which can prevent platelet aggregation and also disaggregate platelet clumps. The lungs can also degrade the potent, platelet aggregating agent adenosine-5'diphosphate (ADP) (5). Thus, in conjunction with our studies on prostaglandins and related substances, we have examined pulmonary artery endothelial cells in culture for ADPase activity. Approximately 10^6 cells were incubated with [^{14}C]- or [^3H]ADP (with or without carrier) at 37°C in 5 ml of culture medium. At timed intervals, the incubation medium was examined by thin-layer

chromatography. ADP at concentrations of $3 \times 10^{-7} \text{M}$ to $3 \times 10^{-5} \text{M}$ was found to have a half-life of about 3.5 minutes. The first product formed was 5'-AMP, which was itself degraded to yield adenosine and inosine. Adenosine did not accumulate in the medium but was taken up by the cells and incorporated into ADP and then into ATP. In a second series of experiments, endothelial cells were removed from their flasks with a rubber policeman and homogenized. Subcellular fractions were prepared by differential centrifugation. ADPase activity occurred most abundantly in the microsomal fraction, enriched in terms of angiotensin converting enzyme (ACE). ACE is known to occur as a component of the plasma membrane of endothelial cells (cf. 23). Thus, pulmonary endothelial cells can prevent intravascular thrombosis by degrading ADP. The ADPase activity is probably a component of the cell membrane.

Synthesis of Angiotensin Converting Enzyme by Endothelial Cells in Culture

Recently we undertook a study to determine whether endothelial cells in culture synthesize angiotensin converting enzyme, or whether they merely provide highly specific receptors for the enzyme. Previously, we have shown that endothelial cells from cow, pig, rabbit, and rat possess angiotensin converting enzyme, and that the enzyme occurs along the plasma membrane and associated caveolae (21, 26, 28). Further, we have shown that the angiotensin converting enzyme activity persists through more than 50 passages (19). Initially, we took these data to mean that endothelial cells in culture are capable of synthesizing angiotensin converting enzyme. However, all of our previous studies used cells grown in medium containing fetal calf serum (FCS) at 20 to 30% v/v. FCS is a rich source of angiotensin converting enzyme; hence, it seemed possible that endothelial cells provide receptors for, but do not synthesize, angiotensin converting enzyme. To resolve this problem, we obtained cultures of bovine pulmonary endothelial cells in FCS previously treated by heating at 56°C , a maneuver reported to inactivate angiotensin converting enzyme (6). The primary cultures were propagated through four passages, such that the average progeny in the fourth generation of each cell in the original isolate was 6,400. The cells were assayed using [^3H]benzoyl-Phe-Ala-Pro as substrate (18). The specificity of the assay was tested by using SQ 14,225 (10^{-5}M and 10^{-6}M), a specific inhibitor of angiotensin converting enzyme.

Enzyme activity per cell was remarkably constant, although there was a tendency for cell size to increase along with, in the fourth generation, an increase of enzyme activity per cell. Under the conditions of these experiments, there was a 12,428-fold increase in angiotensin converting enzyme activity. Thus, these results support the concept that bovine pulmonary artery

endothelial cells in culture are capable of specific protein synthesis. Electron micrographs of grazing sections of the cells in culture suggest that the cells appear to have the subcellular machinery (extensive rough endoplasmic reticulum and polyribosomes [Fig. 6]) to synthesize a complex glycoprotein such as angiotensin converting enzyme (23).

CONCLUDING REMARKS

As described above, techniques for the isolation and culture of endothelial cells from bovine pulmonary artery have been established. These cultures have provided useful information on specific metabolic activities of pulmonary endothelium, and have the advantage that large quantities of cells can be obtained in original isolates. In addition, antigenic sites of bovine cells appear to cross-react with antibodies to human antisera. However, the techniques are not suitable for obtaining endothelial cells from small animals. Moreover, endothelial cells of pulmonary artery and those of the microvascular bed of the lungs in situ differ in terms of structure. In addition, the small caliber and large extent of surface area of the pulmonary microcirculation make it likely that processing of blood-borne substrates such as angiotensin, bradykinin, adenine nucleotides and amines takes place at the level of the smallest vessels. We have, therefore, developed a technique for obtaining endothelial cells from the lungs by vascular perfusion with collagenase (500 units/ml) in HEPES buffered salt solution.

Although one can readily isolate and culture endothelial cells from pulmonary artery of, for example, cow (23, 26, 28), and one can readily isolate and culture endothelial cells from human umbilical vein (12, 19), we believe that our newly developed techniques for obtaining endothelium from the lungs of small animals by vascular perfusion may have at least two effects that will accelerate the pace and broaden the scope of studies on specific metabolic functions of the lungs. First, the vast preponderance of studies on the possible metabolic functions of the lungs have been performed with small animals, in which it is virtually impossible to obtain an adequate number of cells from main-stem pulmonary artery. Second, although we and others have shown that cells from main-stem pulmonary artery are capable of metabolizing bradykinin, angiotensin, ADP, adenosine, and biogenic amines, the general assumption is that the processing of such substances in vivo occurs most prominently at the level of the microcirculation. Our new techniques of cell isolation allow use of small animals and therefore allow a more direct comparison with previous functional studies in which small animals, such as rats and rabbits, have been used.

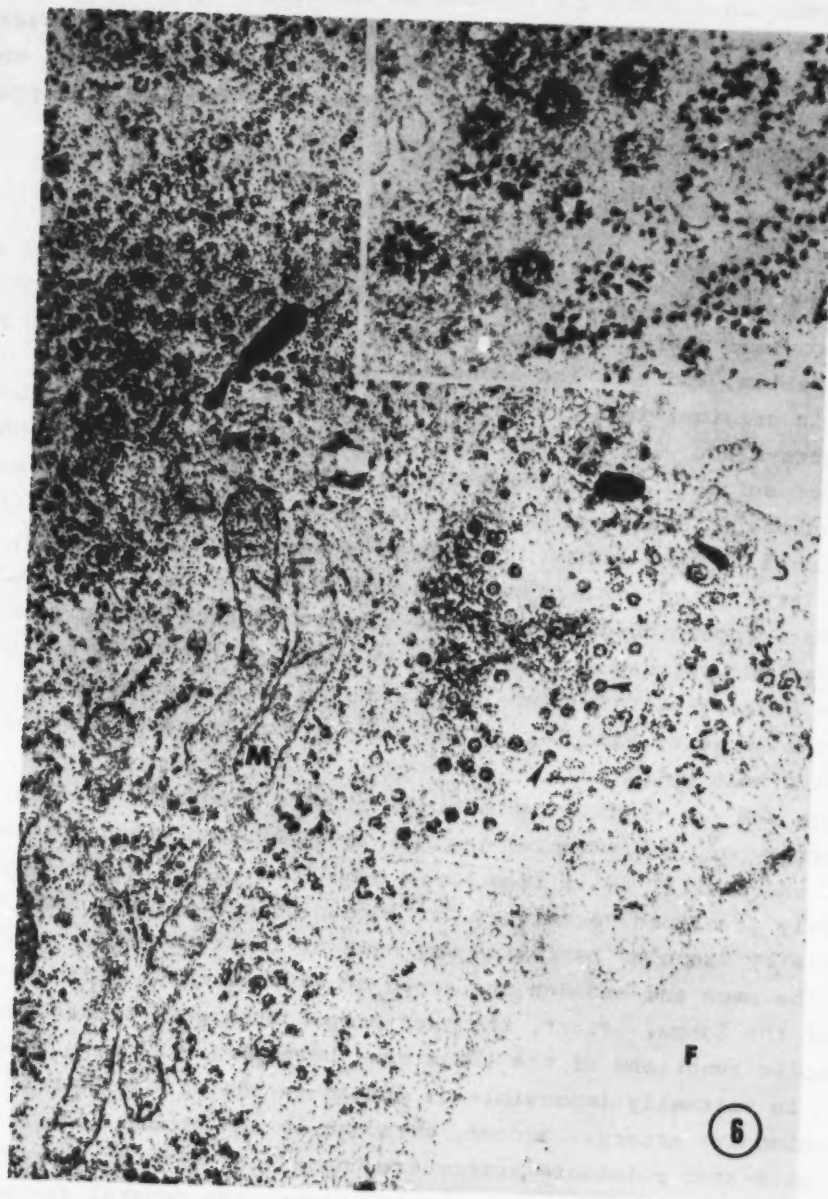


Figure 6. En face section of calf pulmonary endothelial cell spanning the cell from the culture flask (F) to the nucleus. The plane of section has grazed the nuclear envelope revealing nuclear pores (arrowheads) and also grazed the plasma membrane revealing abundant caveolae (*). In addition to elongated mitochondria (M), the cytoplasm contains large numbers of whorled polyribosomes (arrows) attached to the cisternae of the endoplasmic reticulum. The insert shows an enlargement of a portion of the field showing the spiral arrangement of ribosome complexes. X22,000; insert X75,000. Reproduced with permission from *Tissue & Cell* 10:535-554, 1978.

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DISCUSSION

PARTICIPANT: Could you tell me the evidence that the endothelial cells in vivo synthesize angiotensin converting enzyme, rather than picking it up from the blood?

DR. U. RYAN: The best evidence is that they're actually synthesizing. Of course, the serum enzymes have got to come from somewhere. James probably can be a little more certain on this. The serum enzymes may well have come from the lungs.

DR. J. RYAN: That's somewhat suggestive, but what is pretty clear is that the serum enzyme differs molecularly from the lung enzyme. It has extra sugars. That's not the result you'd expect that the enzyme is part of the serum.

PARTICIPANT: The other possibility is that the synthesis is in the liver, and that the sugars are split off into the pulmonary endothelial cell membrane, and then the enzyme is absorbed. But that doesn't seem to be the case.

DR. J. RYAN: The cells in culture certainly make the enzyme; there's no question about that. Liver has a remarkably little angiotensin converting enzyme, but that's not quite the same as saying that it might possibly make it very fast. Our prejudice is otherwise.

DR. BROMBERG: Where does the enzyme come from in sarcoidosis?

DR. U. RYAN: I don't know. I don't think the evidence is that good that it comes from macrophages. I don't see why it couldn't be endothelial cells, but we don't know.

DR. J. RYAN: Let's ask that question of you. What percentage of these patients have vasculitis?

DR. BROMBERG: According to the International Congress at the New York Academy about three years ago, it was listed in three papers as appearing in severe sarcoidosis, but I guess that there's some dissent.

DR. U. RYAN: We began a study on that, but the biopsy material was very hard to analyze. If you think about these pictures, that's an incredibly fine localization which requires extremely good fixation.

Divergent Effects of Prostaglandins on the Pulmonary Vascular Bed

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In most organ systems including the lung, arachidonic acid (5,8,11,14-licosatetraenoic acid) is converted into the endoperoxide intermediates PGG_2 and PGH_2 by a microsomal cyclooxygenase (7, 8, 22). The endoperoxide intermediates (PGG_2 and PGH_2) are then converted by terminal enzymes into primary prostaglandins (PG), thromboxane A_2 (TXA_2), or prostacyclin, PGI_2 (1, 5, 6, 8, 9, 21-23). The distribution and activity of terminal enzymes determine the pattern of products formed from endoperoxide intermediates in an organ (10, 11, 23). Many reports indicate that the endoperoxide intermediate, endoperoxide analogs, PGE_2 , $\text{PGF}_{2\alpha}$ and PGD_2 all increase pulmonary vascular resistance in a variety of species (10, 12, 14-17, 27). In contrast, PGE_2 has dilator activity in the pulmonary circulation of fetal and neonatal animals (3). The pulmonary vascular effects of TXA_2 are uncertain, but this labile substance has potent smooth muscle stimulating and platelet aggregating activity and its breakdown product, TXB_2 , has modest pressor activity in the pulmonary vascular bed (9, 18). In contrast to the effects of PGH_2 , PGE_2 , PGD_2 , $\text{PGF}_{2\alpha}$ and TXB_2 , the newly discovered metabolite of arachidonic acid metabolism, PGI_2 , has pulmonary vasodilator activity (9, 18). However, the effects of arachidonic acid on the pulmonary vascular bed are unclear. Arachidonic acid has been shown to increase pulmonary vascular resistance in a number of species when injected as a bolus and to contract isolated segments of pulmonary artery (4, 10, 12, 14, 25, 28). These responses are blocked by indomethacin, suggesting that in the lung arachidonate, when administered as a bolus, is converted to substances that have pulmonary vasoconstrictor and bronchoconstrictor activity

(10, 12, 14, 25, 28). It has, however, recently been reported that arachidonic acid has depressor activity in the pulmonary circulation when the prostaglandin precursor is infused (12). It has also been recently shown that the newly discovered bicyclic prostaglandin, PGI_2 , dilates feline and canine pulmonary vascular beds (13, 19). The purpose of the present report is to compare responses to primary prostaglandins (PGE_2 , $\text{PGF}_{2\alpha}$ and PGD_2), arachidonic acid, and prostacyclin in the canine pulmonary vascular bed and the airways.

METHODS

The pulmonary vascular effects of primary prostaglandins, arachidonic acid, and prostacyclin were investigated in mongrel dogs unselected as to sex and weighing 14.2-24.5 kg. The dogs were anesthetized with pentobarbital sodium (30 mg/kg, iv) and strapped to a Philips heart table in the supine position. A 6F Edslab thermal dilution catheter was passed into the main pulmonary artery from the external jugular vein under fluoroscopic guidance (Philips image intensifier). Pulmonary arterial pressure was measured from the distal port on the Edslab catheter. A 7F Teflon catheter was passed into the left atrium transeptally and large bore catheters were positioned in the aorta from a femoral artery and in a femoral vein. Cardiac output was determined with an Edwards thermal dilution computer, model 9500, after injection of 5 ml of 5% dextrose solution (cooled to 0°C) into the superior vena cava (proximal port on the Edslab catheter). Values for cardiac output averaged 120 ml/kg per minute and compared favorably with cardiac outputs determined by the indicator-dilution technique in this laboratory. The dogs breathed room air or room air enriched with O_2 spontaneously through a cuffed endotracheal tube.

In dogs in which constant flow perfusion of the left lower lobe was employed, a specially designed 20F double-lumen balloon catheter was introduced through a jugular vein into the arterial branch of the left lower lung lobe under fluoroscopic guidance. A 1.5 mm Teflon catheter with its tip positioned about 2 cm distal to the tip of the perfusion catheter was used to monitor perfusion pressure in the lobar artery. Catheters with side holes near the tip were passed into the main pulmonary artery and femoral artery and transeptally into a small intrapulmonary vein and the left atrium. Precautions were taken to ensure that pressure measurements were made without wedging in veins 2-3 mm in diameter. Briefly, a 0.9 mm Teflon catheter with two side holes near the tip was passed through a 3 mm Teflon catheter that previously had been wedged in a small intrapulmonary vein. The 0.9 mm catheter was then withdrawn 1-3 cm from the wedge position until pressure dropped abruptly. The 0.9 mm catheter was fixed in place with a Cope adaptor after the larger catheter had been withdrawn to the left atrium. These methods have been described in

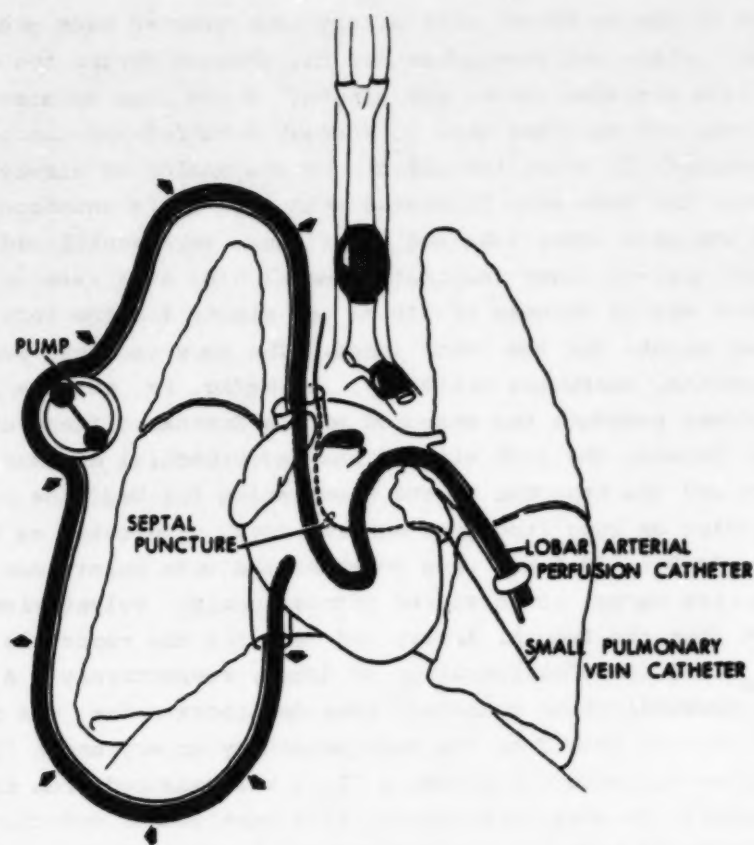


Figure 1. Diagram showing catheterization procedure in the dog. A specially designed 20F balloon catheter is passed into the artery of the lower left lobe and the lung is autoperfused with the blood withdrawn from the right atrium. Vascular pressures are measured in the perfused lobar artery, a small pulmonary lobar vein, and the left atrium and in the main pulmonary artery and the aorta. The Carlen's endobronchial catheter permits the left lower and right lungs to be ventilated separately.

detail previously, and a diagram of the catheterization procedure is shown in Figure 1 (12, 15). All vascular pressures were measured with Statham P23D transducers zeroed at the level of the middle of the right atrium, and mean pressures were recorded on an oscilloscopic recorder (model DR-12, Electronics for Medicine). After all catheters had been positioned and the dogs heparinized (500 U/kg, iv) the balloon on the perfusion catheter was distended with 2-4 ml of 50% sodium diatrizoate (Hypaque Winthrop) until pressure in the lobar artery and small vein decreased to near left atrial pressure. The vascularly isolated left lower lobe then was perfused with a Sarns roller pump (model 3500) with blood withdrawn from the right atrium. The pumping rate was adjusted so that

mean pressure in the perfused lobar artery approximated mean pressure in the main pulmonary artery and thereafter was not changed during the experiment. The pumping rate averaged 260 ml per minute. These dogs spontaneously breathed room air or room air enriched with O_2 through a cuffed endotracheal tube.

In experiments in which the effects of the analog on airway pressure were evaluated, the dogs were intubated with a Carlen's endobronchial divider (No. 39) and the left lower lobe and right lungs were ventilated separately with a Harvard dual-cylinder respirator (model 618) at a rate of 20 cycles per minute with stroke volumes of 110 ml per minute for the left lower lobe and 280 ml per minute for the right lungs. The dogs received succinylcholine chloride (Anectine, Burroughs Wellcome), 2.5 mg/kg, iv, to paralyze ventilation. Translobar airway pressure was measured with a Satham differential transducer (PM5) bridged between the left side of the endobronchial divider and the pleural space and was recorded on the Electronics for Medicine recorder.

For studies on lung function, mongrel dogs, unselected as to sex and weighing from 14.2 to 18.5 kg, were anesthetized with chloralose (50 mg/kg) and urethane (500 mg/kg) administered intravenously. Polyethylene catheters were advanced from the femoral artery and vein for the recording of aortic pressure (P_{Ao}) and the administration of drugs, respectively. A 6F Edslab double lumen thermodilution catheter (Edwards Laboratories) was passed from the external jugular vein into the main pulmonary artery under fluoroscopic guidance. Pulmonary arterial pressure (P_{PA}) was measured from the distal port of this catheter. In some experiments, left ventricular end-diastolic pressure was measured through a Cordis pig-tail catheter advanced from a femoral artery or, alternately, left atrial pressure was measured directly through a 7F Teflon catheter positioned transeptally in the left atrium. Cardiac output was determined with an Edwards Laboratories Thermal Dilution Computer, Model 9500A. All vascular pressures were measured with Satham P23BB or P23AC transducers zeroed at atrial level. Mean pressures were obtained from the pulsatile signal by electrical averaging.

The dogs were ventilated with a Harvard ventilator through a short tracheal cannula (2-3 cm diameter) introduced by tracheostomy. Transpulmonary pressure (P_{TP}) was measured by a Satham PM5E differential transducer interposed between the tracheal cannula and a Harvard pleural cannula inserted through the chest wall at the 4th or 5th intercostal space. Pneumothorax was adjusted to 10-20 ml immediately after introduction of the pleural cannula. Air flow, ($\dot{V}$), was measured with a Fleisch No. 1 pneumotachograph heated above body temperature and coupled to a Grass PT5A differential transducer. P_{TP} and $\dot{V}$ signals were processed by a Hewlett-Packard 8816A Respiratory Analyzer which provided, on line, volume (V), dynamic compliance (C_{dyn}), and resistance of the lung (R_L). C_{dyn} was computed between points of zero flow by dividing the volume of each breath by the difference between end-inspiratory and end-expiratory P_{TP} . R_L

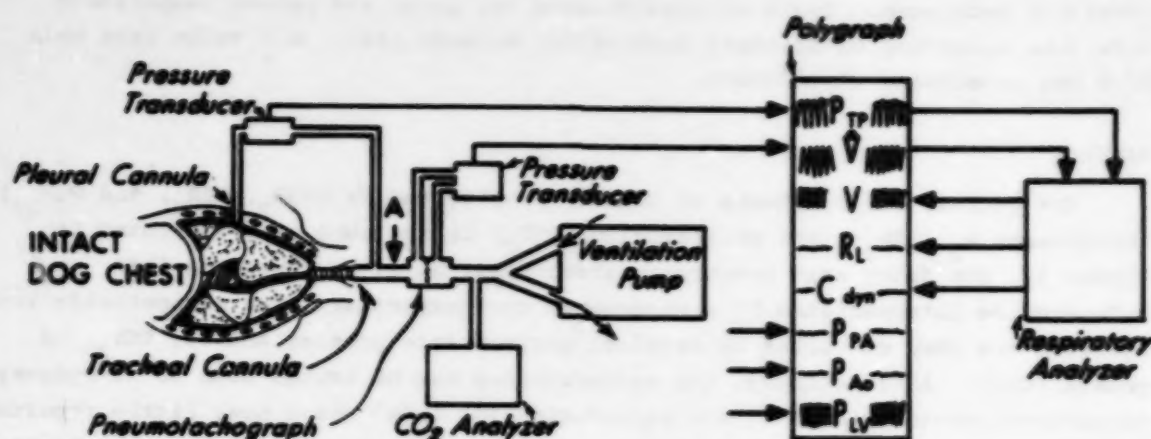


Figure 2. Schematic diagram of the procedure used to evaluate the effects of the prostanooids on lung function in a dog with an intact chest.

was computed at early expiration by the method of Mead and Whittenberger (25) in which instantaneous resistive pressure was divided by instantaneous flow. Resistive pressure is derived by subtracting the ratio of volume to computed compliance from P_{TP} . Percent CO_2 in end-tidal air was monitored periodically with a Beckman LB-2 medical gas analyzer connected to a tap between the respirator and the pneumotachograph. P_{TP} , $\dot{V}$, V , R_L , C_{dyn} and vascular pressures were recorded on a Grass model 7C 8-channel recorder. The preparation used to investigate the effects of the prostaglandins on lung function in a dog with an intact chest is illustrated in Figure 2, and this preparation has been described previously (25). The animals were heparinized with sodium heparin (Upjohn), 500 units/kg iv, and spontaneous breathing was arrested with succinylcholine chloride (Anectine, Burroughs Wellcome), 30-40 mg iv, repeated as needed. After neuromuscular blockade, increments of anesthetic were given as needed. Minute volume was set with tidal volume at 12 ml/kg and the rate sufficient to maintain end-tidal CO_2 near 5% and blood gases in a normal range. A lung volume history was established by hyperinflating the dog to 3 times the tidal volume 3 minutes prior to each administration of test agent into the superior vena cava (SVC).

Arachidonic acid (NuChek), 99% pure, and indomethacin (Merck) were freshly prepared as sodium salts: arachidonic acid in 10% ethanol in 100 mM sodium carbonate and indomethacin in 100 mM sodium carbonate in normal saline. Prostaglandins D_2 , E_2 , and $F_{2\alpha}$, and the PGH_2 analog (Upjohn) were dissolved in absolute ethanol and stored at $-20^\circ C$. Working solutions were freshly prepared in saline. PGI_2 was prepared in 20 mM Tris buffer, pH 8.5, and was stored in a freezer.

All values are expressed as the mean $\pm$ standard error of the mean unless

otherwise indicated. Tests of significance for group and paired comparisons were done according to standard statistical methods (24). A P value less than 0.05 was considered significant.

RESULTS

The pathway for synthesis of primary prostaglandins (PGD_2 , PGE_2 , and $\text{PGF}_{2\alpha}$), thromboxane A_2 (TXA_2), and prostacyclin (PGI_2) in the lung is illustrated in Figure 3. The fatty acid precursor, arachidonic acid, is converted into endoperoxide intermediates by a microsomal cyclooxygenase. The endoperoxide intermediates are then converted by terminal enzymes into prostaglandins, TXA_2 , or prostacyclin. Alternatively, the endoperoxides may be broken down to 12-hydroxy-heptadecatrienoic acid (HHT) and malondialdehyde (MDA) which have little reported biologic activity. Although the endogenous precursor is derived from the breakdown of phospholipids in cell membranes, exogenous arachidonic acid can also serve as substrate for the cyclooxygenase. The effects of exogenously administered arachidonic acid on the pulmonary vascular bed perfused at constant flow are illustrated in Figure 4. Injection of 3 mg arachidonic acid, as a rapid bolus into the perfused lobar artery, produced a rapid increase in lobar arterial and small vein pressures but had no effect on left atrial pressure. The effects of arachidonic acid on lobar arterial and venous pressure were dose-related in the

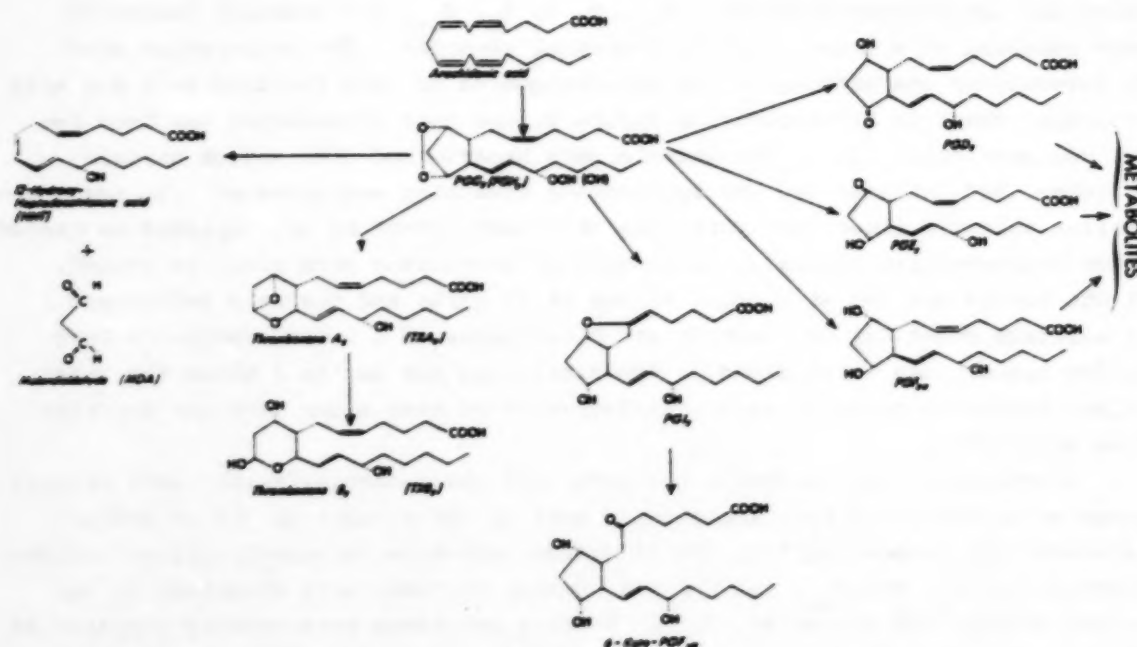


Figure 3. Diagram showing the pathway for biosynthesis of PGD_2 , $\text{PGF}_{2\alpha}$, PGE_2 , thromboxane A_2 , and PGI_2 in the lung.

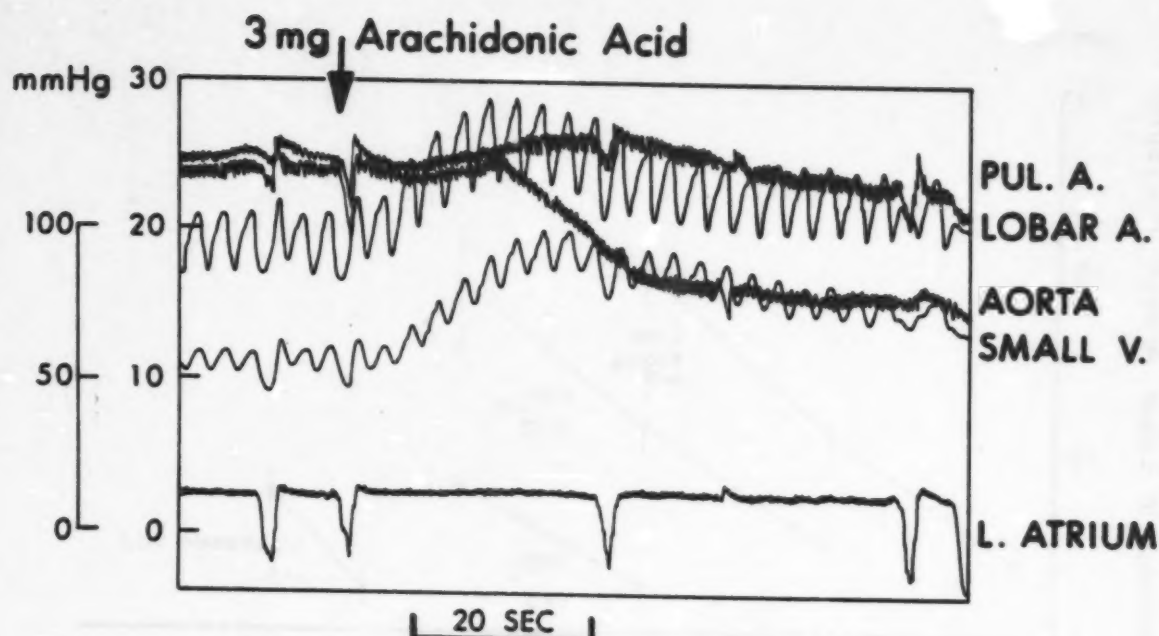


Figure 4. Records from an experiment in a dog with an intact chest, showing the effects of arachidonic acid (3 mg) injected into the lobar artery on mean pressures in the main pulmonary artery (Pul. A.), the lobar artery (Lobar A.), the aorta, the small intrapulmonary vein (Small V.), and the left atrium (L. Atrium). Blood flow to the left lower lobe was maintained constant with a pump.

range of doses of 0.1-3 mg, and at doses of 1-3 mg aortic pressure was decreased.

The pressor activities of arachidonic acid, PGE_2 , $\text{PGF}_{2\alpha}$, and a stable endoperoxide (PGH_2) analog in the pulmonary vascular bed under conditions of controlled flow are compared in Figure 5. $\text{PGF}_{2\alpha}$ is about 10-fold more potent than PGE_2 which in turn is about 10-20 times more active than arachidonic acid. The stable endoperoxide analog is about 10-fold more active than $\text{PGF}_{2\alpha}$. Although not shown in Figure 5, the pressor activities of $\text{PGF}_{2\alpha}$ and PGD_2 are very similar. In addition to increasing lobar arterial and venous pressures, arachidonic acid increases translobar airway pressure, indicating that the prostaglandin precursor is converted into substances that have bronchomotor activity in the dog (Fig. 6). The airway effects of arachidonic acid were studied further using the procedure illustrated in Figure 2. The effects of arachidonic acid on the airways are depicted in Figure 7. Rapid bolus injection of 10 mg of the prostaglandin precursor into the superior vena cava increased transpulmonary pressure to 200% of control value. The response attained a peak within 20 seconds, and transpulmonary pressure gradually returned toward control value. The rise in transpulmonary pressure was accompanied by

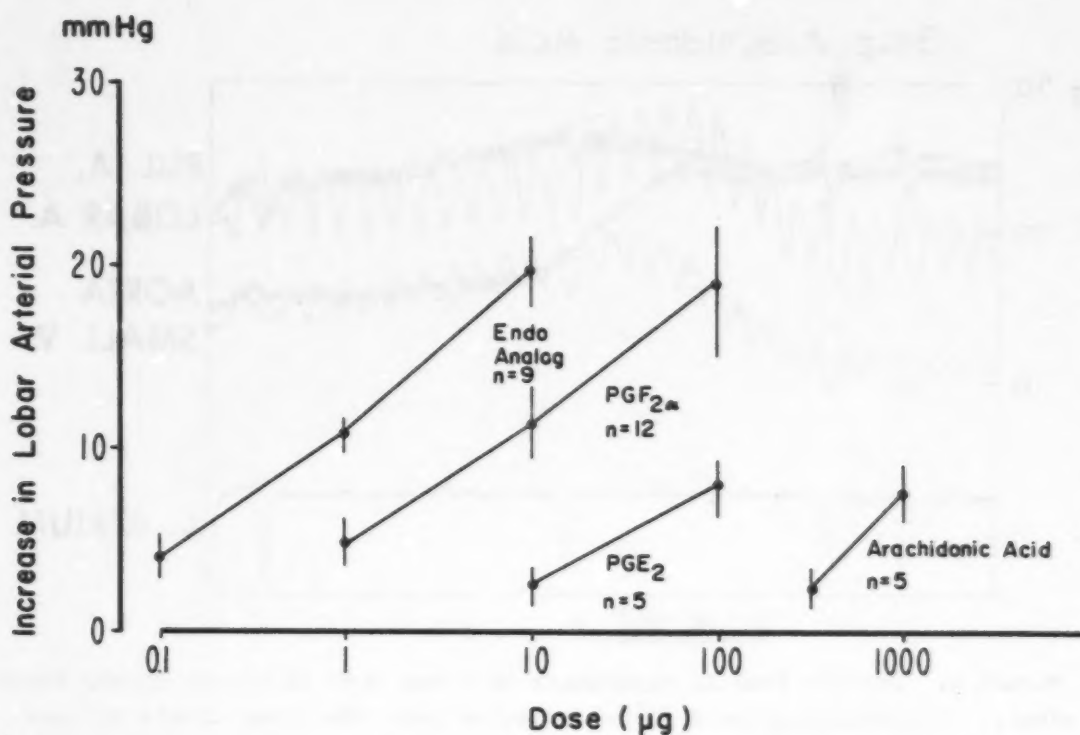


Figure 5. Dose-response curves comparing increases in lobar arterial pressure in response to graded doses of an endoperoxide (PGH_2) analog, $\text{PGF}_{2\alpha}$, PGE_2 , and arachidonic acid. N indicates number of animals studied. Blood flow to the left lower lobe was maintained constant with a pump.

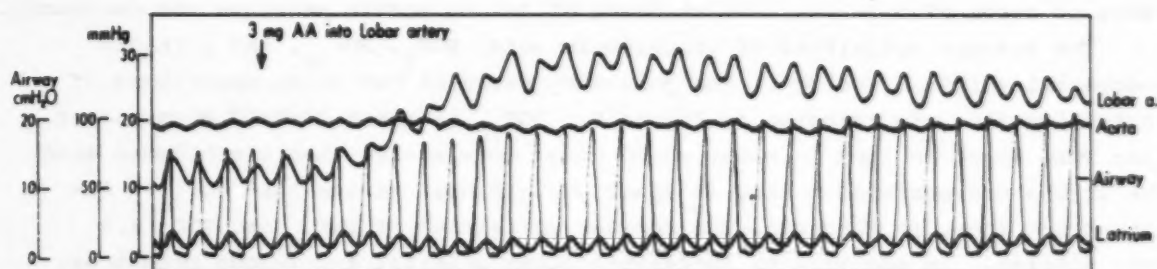


Figure 6. Tracings from an experiment illustrating the effects of injection of arachidonic acid (3 mg) into the perfused lobar artery on mean vascular pressures in the lobar artery (Lobar a.), the aorta, the left atrium (L. atrium) and on translobar airway pressure. Blood flow to left lower lobe was maintained constant with a pump, and the left lower lobe was ventilated separately with a Carlen's endobronchial divider.

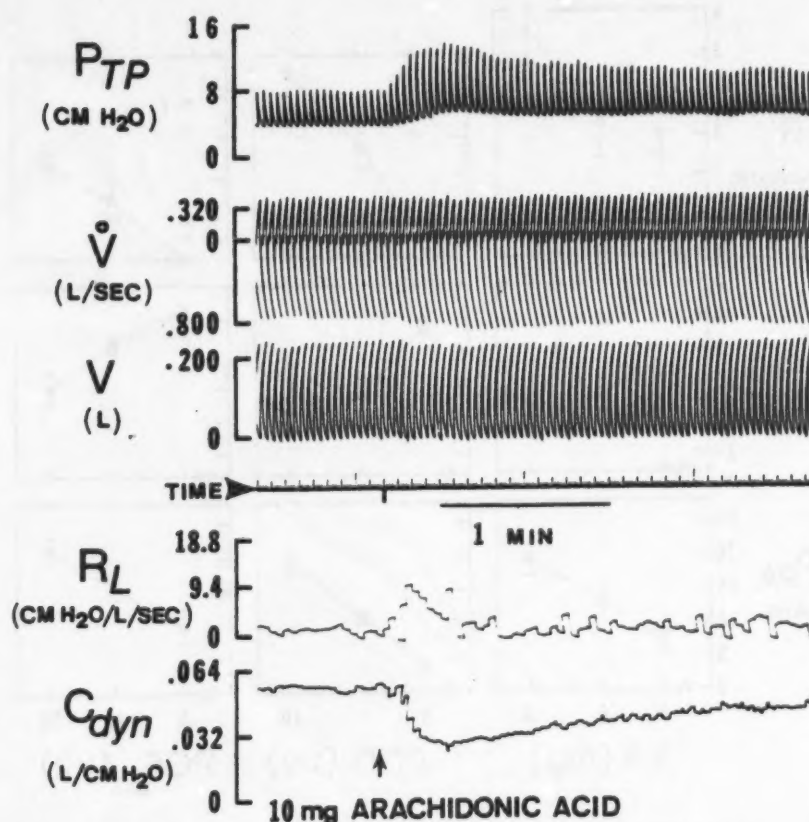


Figure 7. Record from an experiment illustrating the effects of archidonic acid (10 mg) on transpulmonary pressure (P_{TP}) (inspiration up), air flow ($\dot{V}$) (inspiration upward from zero, expiration down), tidal volume (V), lung resistance (R_L), dynamic compliance (C_{dyn}) in a dog with an intact chest. Mean pulmonary arterial pressure (P_{PA}) was increased and mean aortic pressure (P_{Ao}) (not shown in this figure) was decreased. Injection was made as a bolus into the superior vena cava at the point indicated.

a transient rise in lung resistance and a greater and more sustained decrease in dynamic compliance. The effects of arachidonic acid on lung function were dose-related, and hyperinflation of the lung to total lung capacity 10-15 minutes after administration of the prostaglandin precursor usually returned lung resistance and dynamic compliance to control levels. The effects of arachidonic acid, PGD_2 , and $PGF_{2\alpha}$ on lung function in the intact dog are compared in Figure 8. All three substances increased pulmonary arterial pressure and lung resistance and decreased dynamic compliance in a dose-dependent manner. PGD_2 was three times more active than $PGF_{2\alpha}$ in increasing lung resistance and decreasing lung compliance, whereas both prostaglandins were more than 1000-fold more active than arachidonic acid. PGE_2 had only small airways effects

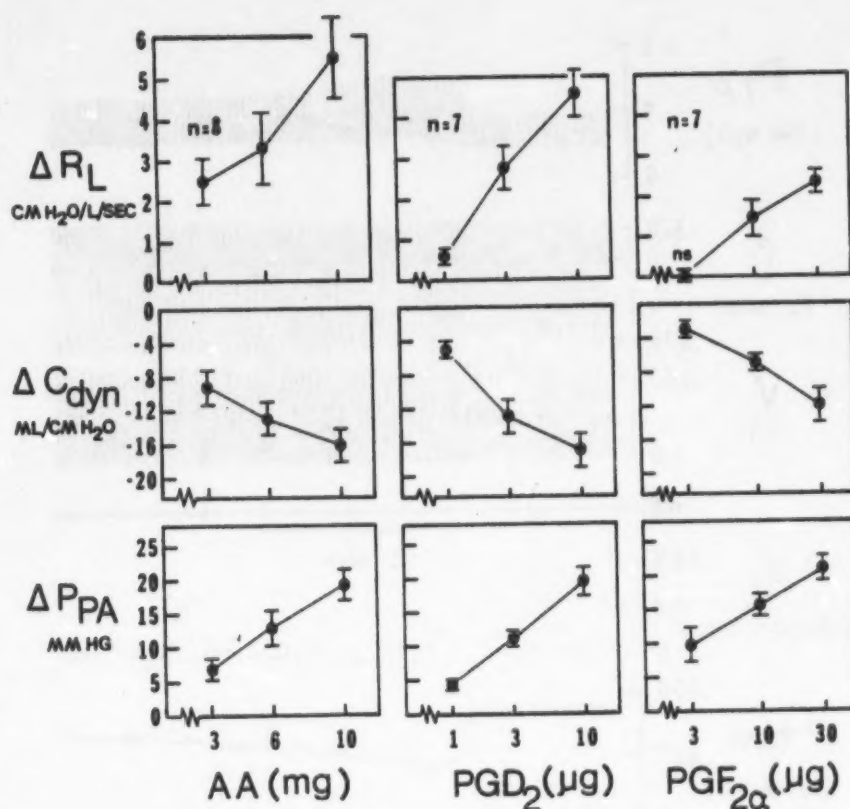


Figure 8. Dose-response curves comparing effects of arachidonic acid (AA), PGD₂, and PGF_{2α} on lung resistance (R_L), dynamic compliance (C_{dyn}), and pulmonary arterial pressure (P_{PA}) in a dog with an intact chest.

in the range of doses of 1-30 μ g. The stable endoperoxide analog and PGD₂ had similar effects on the airways in the intact dog.

The effects of indomethacin on pulmonary vascular, airway, and systemic vascular responses to arachidonic acid are illustrated in Figure 9. The increases in transpulmonary pressure, pulmonary arterial pressure, and aortic pressure were abolished 5-10 minutes after administration of indomethacin, 2.5-5 mg/kg iv. The pulmonary vascular effects of the newly discovered bicyclic prostaglandin, prostacyclin (PGI₂), are summarized in Table 1. PGI₂ produced a small but significant reduction in lobar arterial and small vein pressures when injected as a bolus in doses of 1-10 μ g into the perfused lobar artery. Although responses to PGI₂ were small under resting conditions when tone in the pulmonary vascular bed was minimal, decreases in lobar arterial and venous pressures were substantial when pulmonary vascular resistance was actively increased (Table 1). In preliminary studies PGI₂ had little effect

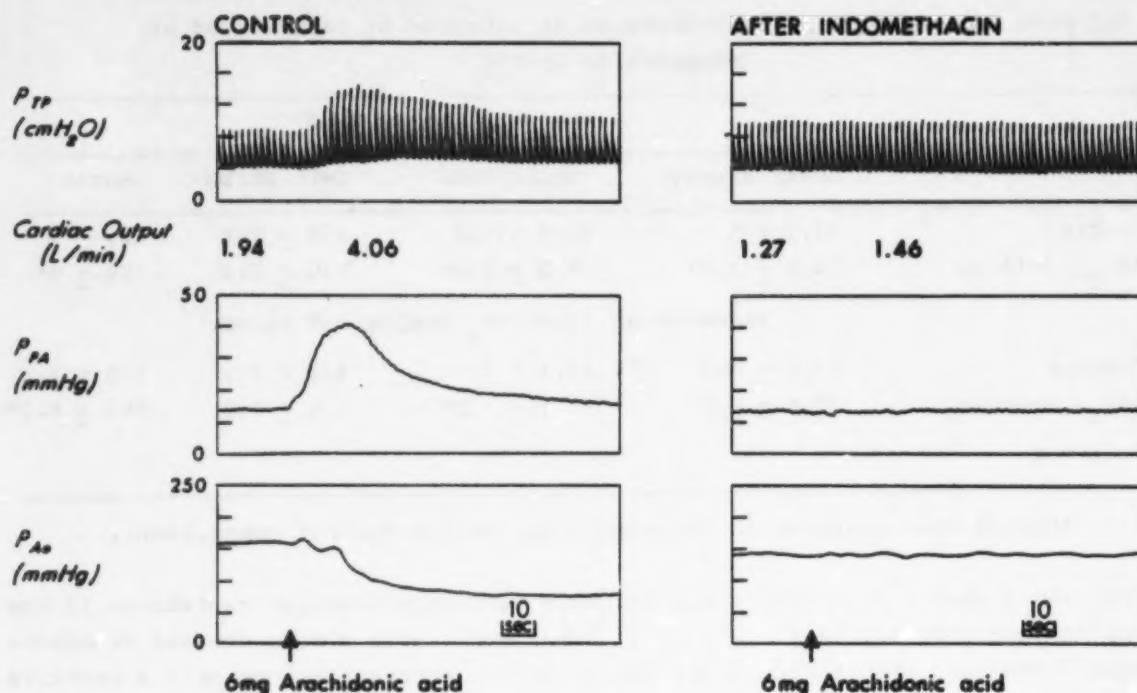


Figure 9. Records from an experiment illustrating the effects of arachidonic acid (6 mg) on transpulmonary pressure (P_{TP}), mean pulmonary arterial pressure (P_{PA}), and mean aortic pressure (P_{AO}) before (panel A) and 20 minutes after (panel B) a slow intravenous infusion of indomethacin, 2.5 mg/kg. At the arrows, arachidonic acid was injected as a bolus into the superior vena cava. Cardiac output in L/min was measured by the thermal dilution technique at the peak of the response.

on the airways in the cat and dog under resting conditions but decreased transpulmonary pressure when bronchomotor tone was enhanced.

DISCUSSION

Results of the present study show that when injected as a bolus the prostaglandin precursor, arachidonic acid, increases lobar arterial and small vein pressures in the intact dog. Inasmuch as pulmonary blood flow was held constant and left atrial pressure was unchanged, the rise in lobar arterial pressure reflects an increase in pulmonary vascular resistance. The rise in small vein pressure and in pressure gradient from the lobar artery to the small vein suggests that arachidonic acid increases pulmonary vascular resistance by constricting small intrapulmonary veins and upstream segments believed to be small arteries (12). The primary prostaglandins (PGE_2 , PDG_2 , and $PGF_{2\alpha}$) as well as

Table 1. Pulmonary Vasodilator Effects on PGI_2 Under Resting Conditions and When Pulmonary Vascular Resistance Is Enhanced by Infusion of an Endoperoxide Analog

	Pressure (mm Hg $\pm$ SE)			
	Lobar artery	Small vein	Left atrium	Aorta
Control	15.9 $\pm$ 0.8	10.5 $\pm$ 0.6	1.8 $\pm$ 0.4	120 $\pm$ 5
PGI_2 , 1-10 μg	14.0 $\pm$ 1.0*	9.2 $\pm$ 0.6*	1.8 $\pm$ 0.4	99 $\pm$ 8*
Infusion of 11,9 PGH_2 Analog 1-5 $\mu\text{g}/\text{min}$				
Control	31.5 $\pm$ 1.3	21.4 $\pm$ 1.0	1.7 $\pm$ 0.2	125 $\pm$ 1.5
PGI_2 , 1-10 μg	25.1 $\pm$ 2.6*	17.1 $\pm$ 1.3*	1.6 $\pm$ 0.2	106 $\pm$ 5.2*
n = 6				

* $P < 0.05$ when compared to corresponding control (paired comparison).

PGH_2 and a stable PGH_2 analog all increase pulmonary vascular resistance in the dog and cat (10, 12, 14-17, 27, 28). Arachidonic acid also increased transpulmonary pressure when the lung was ventilated at constant volume with a positive pressure ventilator (12). In experiments in which the effects of arachidonic acid on lung function were further investigated, the increase in transpulmonary pressure was associated with a transient rise in lung resistance and a sustained fall in dynamic compliance (25). The endoperoxide analog, $\text{PGF}_{2\alpha}$, and PGD_2 all increased lung resistance and decreased dynamic compliance and in this regard were similar to but much more potent than arachidonic acid (25-27). The effects of PGE_2 on the pulmonary vascular bed and on the airways were modest when this substance was injected in the same doses as PGD_2 or $\text{PGF}_{2\alpha}$ (12, 14, 17, 25). Pulmonary vasoconstrictor, bronchoconstrictor, and systemic vasodepressor responses to arachidonic acid were blocked after administration of indomethacin, a cyclooxygenase inhibitor (10-12, 14, 25). These data suggest that the effects of arachidonic acid on the airways and pulmonary and peripheral vascular beds are due to the conversion of the substrate into vasoactive and bronchoactive metabolites in the cyclooxygenase pathway. The pulmonary vasoconstrictor response to arachidonic acid is associated with increased synthesis of E and F "like" prostaglandins; however, it is not known if PGD_2 or TXB_2 is produced by the lung in the intact dog (12). The effects of arachidonic acid on the pulmonary vascular bed were not dependent on the presence of platelets or other formed elements, in that the response to arachidonic acid was not diminished when the lung was perfused with a dextran solution (12). These findings suggest that the substrate is converted to vasoactive substances by the lung itself and that plate-

let aggregation or release of vasoactive products from the platelets plays little or no role in this response (12).

In contrast to the pressor effects of $\text{PGF}_{2\alpha}$, PGD_2 , and PGE_2 or the PGH_2 analog which may mimic the effects of TXA_2 on the pulmonary vascular bed, the newly discovered bicyclic prostaglandin, PGI_2 , had vasodilator activity in the pulmonary vascular bed (13, 19). The pulmonary vasodilator effects of PGI_2 were modest under resting conditions but were greatly enhanced when pulmonary vascular resistance was increased actively by infusion of a vasoconstrictor substance (13). Although PGI_2 had vasodilator activity, arachidonic acid, when administered as a bolus, consistently increased pulmonary vascular resistance in the intact dog and cat and in the isolated dog lung (10, 12, 14, 25, 28). These results suggest that under physiologic conditions in the intact state, the predominant products formed in the lungs when arachidonate is injected as a bolus are vasoconstrictor in nature. Alternatively, it is possible that both vasoconstrictor and vasodilator metabolites are formed but that the activity of the vasoconstrictors overshadows the action of any simultaneously formed PGI_2 "like" substances. It has been reported that PGI_2 is the predominant metabolite formed from arachidonic acid and endoperoxide intermediates in vascular tissue (2, 5, 21). Indeed, we have observed in some animals that slow infusions of arachidonic acid decrease pulmonary vascular resistance in the intact dog and cat. The explanations for the divergent responses to rapidly injected arachidonic acid and slowly infused arachidonic acid are uncertain at the present time. It is, however, possible that when excessive amounts of substrate are converted to PGH_2 , the endothelial prostacyclin synthetase may be overwhelmed and the endoperoxide may isomerize to PGD_2 and PGE_2 or be reduced to $\text{PGF}_{2\alpha}$.

We have reported that administration of cyclooxygenase inhibitors such as indomethacin and melofenamate results in a slow gradual increase in pulmonary vascular resistance in the intact dog (20). It has been shown that a PGI_2 "like" substance is continually released by the lung (6). We have, therefore, suggested that under resting conditions, the pulmonary vascular bed is maintained in a dilated state by production of a vasodilator product in the cyclooxygenase pathway (20). Recent evidence suggests that this vasodilator product in the cyclooxygenase pathway is a PGI_2 "like" substance (6, 13, 19). It would therefore be of interest to ascertain if industrial pollutants such as NO_2 or SO_2 have adverse effects on enzymes such as cyclooxygenase or prostacyclin synthetase which function to synthesize PGI_2 which serves to maintain the pulmonary vascular bed in a dilated state under resting conditions.

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DISCUSSION

DR. WASSERMAN: Have you looked at the effects of PGI_2 on compliance and resistance changes as well?

DR. KADOWITZ: Yes, the dog is a poor model even when you have a marked increase in bronchomotor tone; the infusion of aerosol of PGI_2 doesn't seem to have much effect. The way we can see the effect of PGI_2 is by administering the substance before the bronchial constrictive challenge. If you administer PGI_2 and then follow with a histamine aerosol, the bronchial constrictive response will be markedly attenuated. But, when you administer it when the bronchial motor tone is elevated, you don't see the rapid kind of reversal that we see in the pulmonary vascular bed, and this same kind of thing has been observed by several other people.

Environmental Influences on Uptake of Serotonin and Other Amines

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CLEARANCE OF SEROTONIN AND OTHER AMINES BY THE LUNG

It is well established that the lungs of mammalian species remove 5-hydroxytryptamine (5-HT or serotonin) from the pulmonary circulation and metabolize it to 5-hydroxyindoleacetic acid (5-HIAA) (2, 14, 15, 19, 20, 24). This process of uptake and metabolism serves to inactivate circulating serotonin, and may be important in regulating the systemic arterial concentration of this powerful vasoconstrictor. Although other organs may also remove serotonin from the perfusate, the role of the lung may be of major importance because this organ consists of a vast capillary bed which receives the entire cardiac output. The site of serotonin uptake in the pulmonary capillaries has been localized to the pulmonary endothelial cell (17, 25).

Several lines of evidence indicate that capillary endothelial uptake of serotonin is carrier-mediated, and is very likely an active transport process. First, measurement of 5-HT uptake as a function of perfusate concentration suggests a saturable process (17, 19, 22). Second, uptake requires the presence of Na^+ in the perfusate (19) and is inhibited by ouabain (19, 21), suggesting involvement of the $\text{Na}^+ - \text{K}^+$ activated ATPase. Third, uptake is inhibited by hypothermia (15, 17, 19), anoxia (19, 24), cyanide (24), and absence of a metabolizable substrate (24), indicating the requirement for metabolically generated energy. The use of 2-deoxy-glucose illustrates the requirement for metabolic energy. This agent, which serves as an ATP trap and inhibitor of glucose metabolism, leads to depression of serotonin uptake that can be reversed by

adding a metabolizable substrate (13). Finally, uptake of serotonin can be blocked by inhibitors of amine transport such as imipramine, chlorpromazine, and cocaine (16, 19, 24). Definitive proof that transport occurs by active transport requires demonstration of uptake against a concentration gradient, but this has not yet been accomplished because of the difficulty of demonstrating free, i.e., unbound, intracellular serotonin.

The rate-limiting step for serotonin clearance from the perfusate is the uptake process rather than the subsequent metabolism of 5-HT to 5-HIAA. Thus the presence of a monoamine oxidase (MAO) inhibitor such as iproniazide does not affect the rate of uptake of 5-HT although conversion to 5-HIAA is markedly depressed (2, 14, 15, 19, 24). On the other hand, a possible effect of prolonged MAO inhibition on serotonin uptake has not been evaluated.

Other amines are also removed from the circulation by lung endothelium although uptake mechanisms may vary. Norepinephrine, like serotonin, is removed by a carrier-mediated process of pulmonary endothelium (14, 17, 21) that can be differentiated pharmacologically from the 5-HT carrier (16). Imipramine, which is not transported intracellularly and does not undergo metabolic transformation, is removed through specific binding to the cell membrane (18).

Studies of serotonin uptake from this laboratory with isolated guinea pig lungs are shown in Figures 1 and 2. Similar results were obtained using isolated rat lungs although the normal rates of serotonin clearance are slightly higher with this latter species. Rats were used for studies of oxygen toxicity since the reaction of their lungs to oxygen is better defined and their lungs are easier to perfuse.

PULMONARY OXYGEN TOXICITY

Exposure of animals to oxygen partial pressures above 0.5 atmospheres absolute (ata), i.e., approximately 350 mm Hg, results in their death with a time course that is a hyperbolic function of inspired oxygen (3, 9). With O₂ partial pressures up to approximately 2.5 ata, the lungs are the predominant site of injury. Electron microscopic studies of oxygen-damaged lungs have shown lung cell injury with destruction most marked in the pulmonary endothelium (1, 10, 20). This information provided the background to investigate the influence of hyperoxia on the subsequent ability of lungs to remove serotonin and other amines from the pulmonary circulation. The goal was to define the early manifestations of hyperoxic damage to pulmonary endothelium.

EFFECTS OF OXYGEN EXPOSURE ON CLEARANCE OF SEROTONIN AND OTHER AMINES

Experimental Procedures

Specific pathogen-free rats weighing 250-300 g were exposed to O₂ for varying

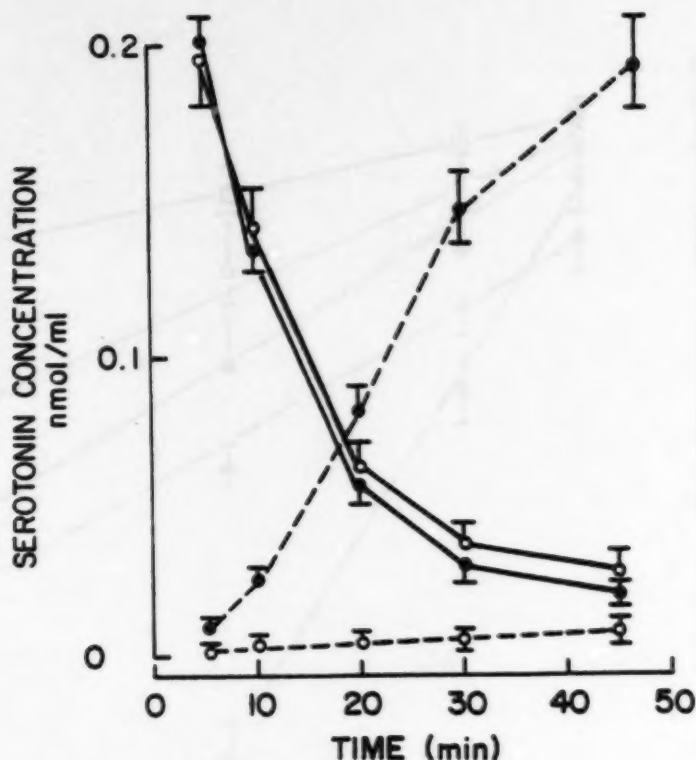


Figure 1. Recirculating perfusion of isolated guinea pig lung with [^{14} C]-serotonin. The concentrations of serotonin (solid lines) and 5-hydroxyindoleacetic acid (dashed lines) are plotted versus time of perfusion. Experiments were carried out in the absence (solid dots) or presence (open dots) of 0.1 mM iproniazide. Results are mean $\pm$ SE for 3 experiments under each condition.

durations at either 1 ata in an environmental chamber or 4 ata in a hyperbaric pressure chamber. The use of specific pathogen-free rats is important to minimize the complication of possible respiratory tract infections. Sexually mature animals were chosen for study because of the known decreased susceptibility of immature rats to the toxic effects of O_2 (26). Ambient CO_2 concentration in the chamber was maintained below 3 mm Hg with CO_2 absorbent. Since rats exposed to O_2 do not maintain food intake, food was withheld from both control and experimental animals to minimize the influence of this variable. Drinking water was provided *ad libitum*. Some animals were maintained on a vitamin E-deficient diet for approximately 6 weeks before O_2 exposure. Vitamin E deficiency in these rats was confirmed with the dialuric acid erythrocyte hemolysis test (6). Rats maintained on the vitamin E-deficient diet gained weight normally.

Following exposure of rats, lungs were removed and evaluated in an isolated lung perfusion system for their ability to remove serotonin, norepinephrine, or

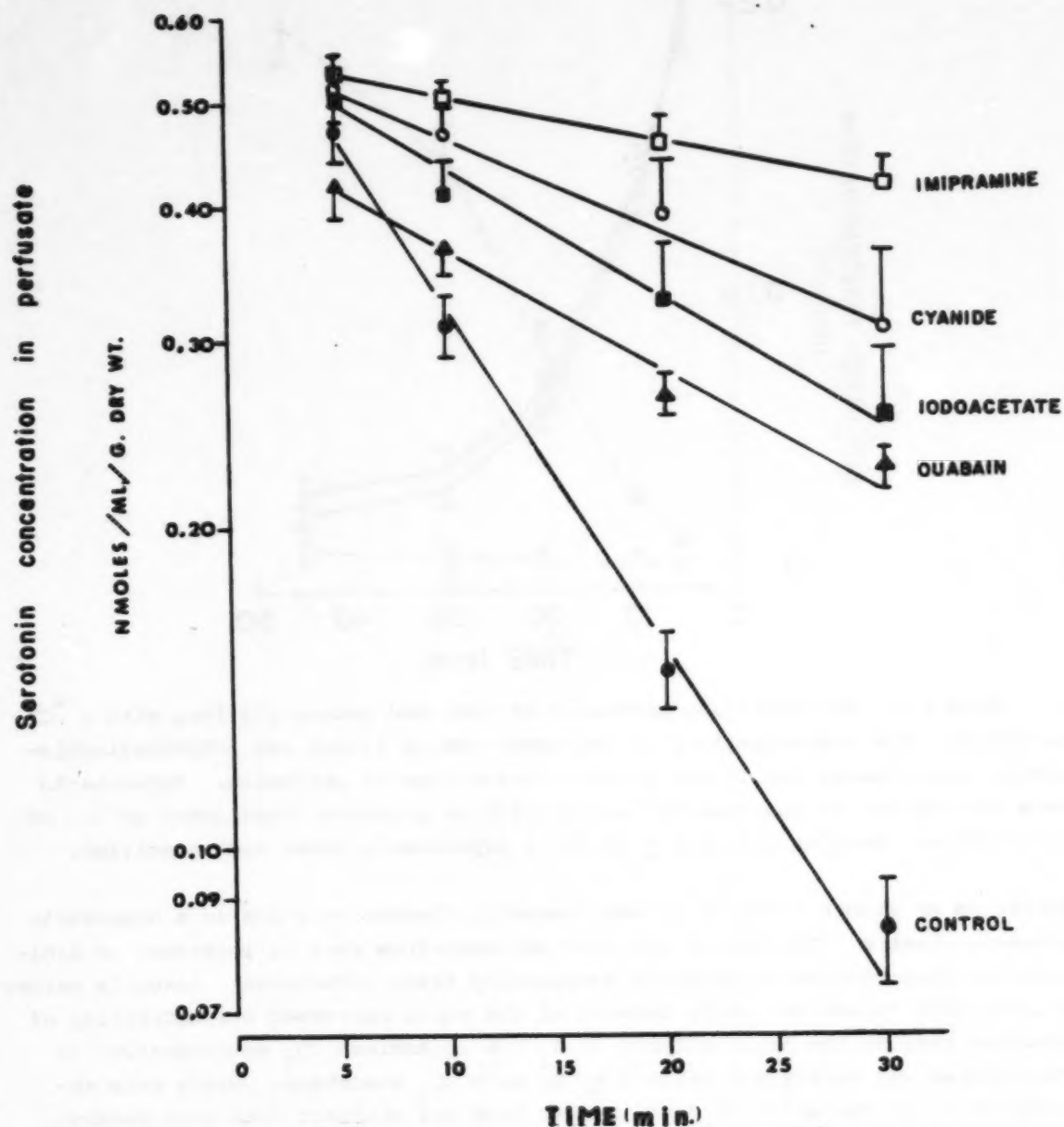


Figure 2. Semi-logarithmic plot of the serotonin concentration in pulmonary perfusate versus time of perfusion of isolated guinea pig lungs in a recirculating system. Serotonin was measured after separation from its metabolic products by column chromatography. The inhibitors used were 0.1 mM imipramine, 1 mM KCN, 1 mM iodoacetate, and 0.01 mM ouabain. Results are the mean $\pm$ SE for 13 control experiments and 3-5 experiments with each inhibitor.

imipramine from the perfusate. Serotonin uptake was evaluated in a recirculating system (12). The first order rate constant was calculated from the semi-logarithmic decrease of perfusate serotonin concentration; the fractional clearance was calculated from the rate constant and the average recirculation time (24). Uptake of norepinephrine and imipramine was evaluated using "once-through" perfusion. Clearance of these amines was calculated from the arterial-venous difference in concentration and the rate of perfusion (5). Assay for each of the amines in the perfusate was done by radiolabel counting following separation of the amine from its metabolic products.

Effects of Oxygen Exposure on Serotonin Clearance

Normal Rats. Fractional clearance of serotonin in control animals that were not exposed to oxygen was approximately 0.80, indicating that approximately 80% of the serotonin presented to the lung was cleared during a single passage through the pulmonary circulation. Mean serotonin clearance in lungs from rats exposed to oxygen was decreased by 5% after 4 hours O_2 , 12% after 12 hours O_2 , 25% after 18 hours O_2 , and 35% after 48 hours O_2 (Fig. 3). The effect of O_2 on serotonin clearance was an approximate hyperbolic function of exposure time with an estimated plateau value of 40-50% depression of clearance. These results suggest that the ability of the lungs to remove serotonin from the perfusate was depressed within the initial 12 hours of oxygen exposure.

Hyperbaric oxygen at 4 ata greatly accelerated the depression of serotonin clearance. After 1 hour of hyperbaric exposure, clearance was depressed by 30% (Fig. 4) which was approximately equivalent to the effect of 1 ata O_2 for 24 hours.

Vitamin E-deficient Rats. Vitamin E-deficient rats demonstrated increased susceptibility to the effects of oxygen on pulmonary clearance of serotonin. With O_2 at 1 ata, 5-HT clearance was depressed by 45% after 12 hours of exposure. This exceeded the effect of 48 hours O_2 exposure on normal animals (Fig. 5). The increased susceptibility to O_2 of vitamin E-deficient rats was also seen with hyperbaric exposure. Serotonin clearance in vitamin E-deficient rats was depressed by approximately 30% after 45 minutes and 45% after 60 minutes of exposure (Fig. 4). Rats repleted with vitamin E by intraperitoneal injection (1 mg/kg twice weekly) or supplementation of drinking water responded to O_2 at 1 ata similarly to normal diet rats. There was no apparent protection against hyperoxia by these pharmacologic doses of vitamin E (6).

Protection Against the Effects of Oxygen. The use of hyperbaric exposure provided a convenient model for evaluating the effect of possible protective agents on the pulmonary response to hyperoxia since only short exposures were required. Rats were treated with a single dose of possible protective agents intraperitoneally 45 minutes prior to exposure (8). GSH (reduced glutathione)

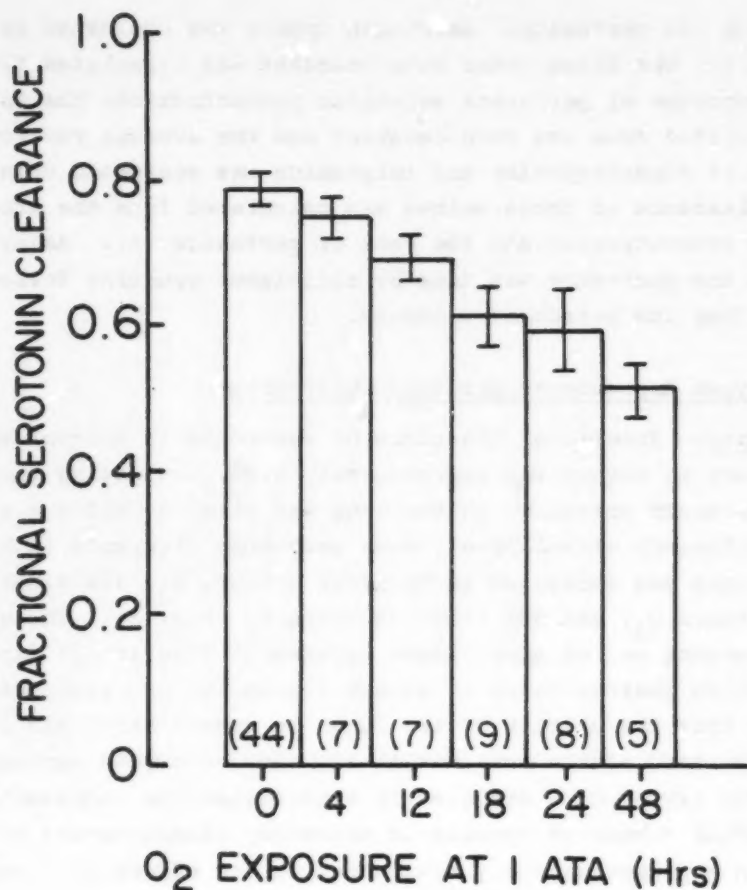


Figure 3. Serotonin clearance by lungs from normal rats exposed to oxygen at 1 atmosphere absolute (ata). Results are the mean $\pm$ SE for the number of experiments indicated at the bottom of each block.

(16 mmol/kg body weight) or sodium succinate (12 mmol/kg body weight) had no effect on subsequent depression of serotonin clearance by hyperbaric oxygen exposure (Fig. 4). It should be noted that these rats were fed prior to administration of succinate although fasting is required to prolong survival by this agent (23). Pretreatment of animals with superoxide dismutase (Palosein^R, Diagnostic Data Inc., Mountain View, CA) (60 nmol/kg body weight) partially prevented the subsequent effects of hyperbaric oxygen on serotonin clearance (Fig. 4). In five animals pretreated with superoxide dismutase, mean serotonin clearance was depressed by only 11% compared with 30% decrease in saline-injected control animals.

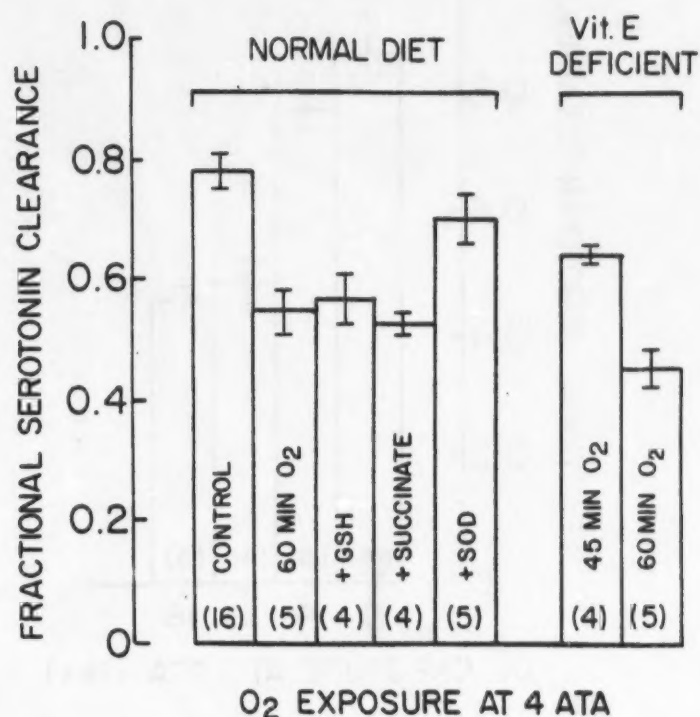


Figure 4. Serotonin clearance by lungs of rats exposed to O₂ at 4 ata. Control animals were maintained in room air. Animals maintained on a normal diet were exposed to O₂ for 1 hour. Vitamin E-deficient animals were exposed to O₂ for 45 or 60 minutes as indicated. Individual groups of animals were pretreated by intraperitoneal injection of GSH (reduced glutathione), succinate, or superoxide dismutase.

Recovery from Hyperoxic Lung Damage. The hyperbaric exposure model was also used to study recovery from oxygen-induced depression of serotonin clearance (7). Following O₂ exposure at 4 ata, animals breathed room air for varying periods before measurement of serotonin clearance. In animals fed a normal diet, serotonin clearance was partially restored to control values after 1.5 hours of air breathing and was approximately 90% of control after 3 hours. On the other hand, recovery of vitamin E-deficient animals from O₂ toxicity was much delayed. Vitamin E-deficient animals showed essentially no recovery of serotonin clearance by 3 hours after O₂ exposure and even 24 hours post-exposure of air breathing, serotonin clearance was 30% below control values. These data suggest that vitamin E not only protects against hyperoxic depression of serotonin clearance but is also required for recovery from the toxic effects of O₂.

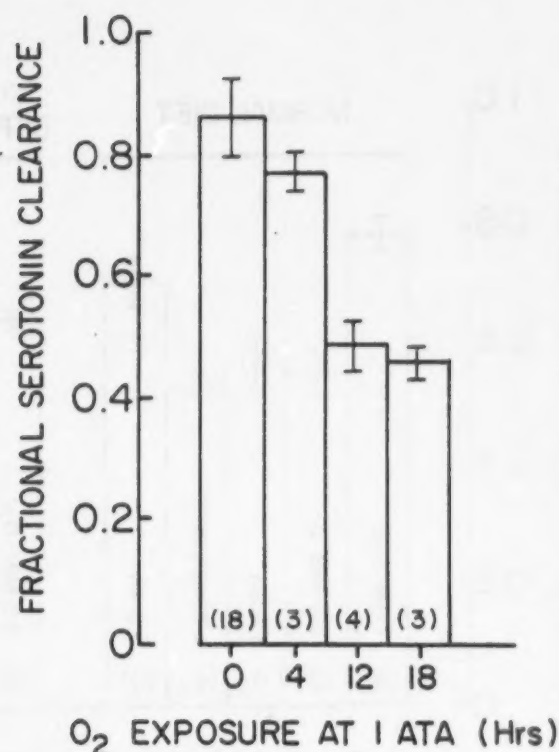


Figure 5. Serotonin clearance by lungs from vitamin E-deficient rats exposed to oxygen at 1 ata for varying durations. Results are mean $\pm$ SE for the number of experiments indicated at the bottom of each block.

Effects of O₂ Exposure on Clearance of Norepinephrine and Imipramine

Clearance of norepinephrine by lungs was also depressed following hyperoxia (5). The relationship between duration of oxygen exposure at 1 ata and depression of norepinephrine clearance essentially paralleled the findings with serotonin (Table 1). Norepinephrine clearance was depressed by 39% after 24 hours and by 51% after 48 hours of O₂. On the other hand, imipramine clearance (4, 5) was unaffected by oxygen exposure for up to 48 hours (Table 1).

PULMONARY PHYSIOLOGIC, MORPHOLOGIC, AND METABOLIC EFFECTS OF HYPEROXIA

The above studies indicated that depression of serotonin uptake was an early and reversible manifestation of pulmonary oxygen toxicity that was potentiated by vitamin E-deficiency. Additional studies indicated that depression of serotonin uptake occurred before other evidence of morphologic, physiologic, or biochemical derangement of the lungs. The normal rats exposed to oxygen at

Table 1. Effect of O₂ Exposure on Lung Clearance of Amines

	Norepinephrine nmol/min/g dry	%*	Imipramine nmol/min/g dry	%*
Control	2.76 ± 0.07 (18)	100	7.52 ± 0.05 (12)	100
O ₂ at 1 ata				
12 hr	2.59 ± 0.22 (8)	94	7.55 ± 0.10 (5)	100
24 hr	1.69 ± 0.14 (8)	61	7.53 ± 0.14 (6)	100
48 hr	1.35 ± 0.21 (6)	49	7.49 ± 0.07 (6)	100

Values are mean ± SE for () experiments.

* Percent of mean control.

1 ata for up to 48 hours or 4 ata for 1 hour had no signs of respiratory distress and their lungs appeared grossly normal. The hyperbaric oxygen exposure was just below the convulsive threshold but an occasional rat did develop generalized seizures and was not used for further studies. Vitamin E-deficient rats developed respiratory distress after 18 hours exposure to 1 ata or 1 hour exposure to 4 ata of oxygen.

Physiologic Changes in Lungs from O₂-exposed Rats

Perfusion pressure at constant flow rate and ventilation pressure at constant tidal volume were measured in the isolated lung during lung perfusion to evaluate lung mechanical and vascular properties. Similar values for these pressure measurements were obtained for control and oxygen-exposed normal or vitamin E-deficient animals. To evaluate the distribution of perfusate to the lung, ⁸⁵Sr-labeled microspheres (15 μ diameter) were infused into the pulmonary artery (6). The distribution of radioactivity per unit tissue was slightly greater in the lower compared to upper lobes, but was similar in control and O₂-exposed animals. The ratio of lung dry to wet weight and the end of perfusion was 0.17-0.18 for both control and oxygen-exposed animals. These are normal values for dry to wet weight ratio of rat lungs perfused with electrolyte solutions, indicating the absence of significant fluid accumulation.

The results suggest that derangement of lung mechanics, altered pulmonary perfusion, or lung edema was not associated with the decreased serotonin clearance that occurred with O₂ exposure.

Metabolic Effects of Hyperoxia

Lung metabolism as a function of oxygen exposure was evaluated by measuring lung tissue adenine nucleotides and the rates of lung lactate and pyruvate production (11). After one hour of perfusion, lung ATP and ATP/ADP were maintained at a high level with no significant difference between control and O₂-exposed rats (Table 2). The rates of production of lactate and pyruvate by the isolated perfused lung and the lactate to pyruvate ratio were unaffected by exposure of rats to oxygen for 18 or 24 hours (11). However, lungs from rats exposed to oxygen for 48 hours demonstrated 60% increase in lactate production with an approximate doubling of the lactate to pyruvate ratio. These studies indicate that, at least through 24 hours of O₂ exposure, there was no alteration of lung glycolysis or energy balance, suggesting that interference with energy generation metabolism was not responsible for altered amine clearance. On the other hand, the possibility of metabolic changes specifically localized to lung endothelial cells cannot be excluded.

Table 2. Effect of O₂ Exposure on Adenine Nucleotide Content of Rat Lungs

	ATP μmol/g dry	ATP/ADP μmol/g dry
Control (3)	12.8 ± 0.1	7.2 ± 0.7
O ₂ at 1 ata		
24 hr (3)	12.2 ± 1.0	8.1 ± 0.4
48 hr (3)	11.8 ± 0.6	7.6 ± 1.1
Control (7)	10.5 ± 0.4	8.6 ± 0.7
O ₂ at 4 ata		
1 hr (4)	9.8 ± 0.4	8.0 ± 0.5

Results are mean ± SE for () experiments.

Table 3. ³H Localization in Autoradiographs of Rat Lung Following Perfusion with [³H]-5-Hydroxytryptamine

	Grains/μ ³		Observed/expected	
	Endothelium	Other	Endothelium	Other
O ₂ , 4 ata x 1 hr	0.09	0.02	1.7	0.3
O ₂ , 4 ata x 1 hr	0.11	0.03	1.6	0.4
O ₂ , 1 ata x 48 hr	0.13	0.03	1.6	0.4

Results are mean values based on 10-15 micrographs.

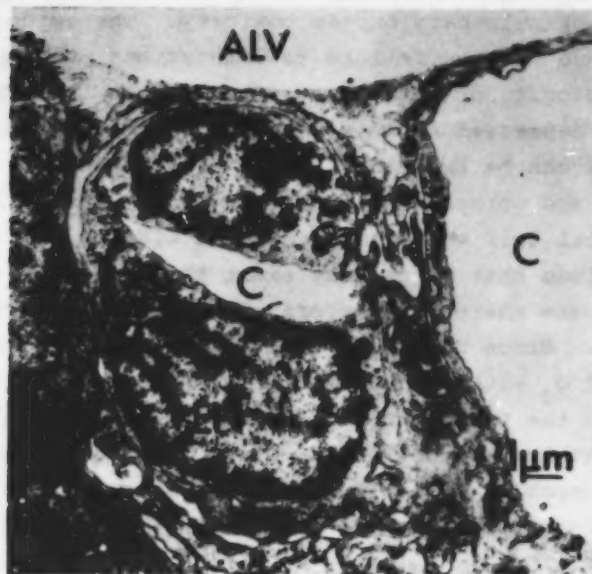


Figure 6. Electron microscope autoradiogram of the lung from a rat exposed to 4 ata O_2 for 1 hour and subsequently perfused with [^3H]-5HT in the presence of iproniazide. Developed silver grains have accumulated predominantly over the capillary endothelium (EN). There are no anatomic alterations in the constituents of the alveolar-capillary membrane attributable to oxygen exposure. Tubular myelin and amorphous material probably representing surfactant are present at the interface between alveolar air and alveolar epithelium. Sections were stained with uranyl acetate-lead hydroxide. ALV, alveolar space; C, capillary space. (Original magnification X6,300).

Electron Microscopy and Autoradiography

In order to study the effect of O_2 on localization of 5-HT, the lungs from two rats exposed to O_2 at 4 ata for 1 hour and one rat exposed to O_2 at 1 ata for 48 hours were perfused with [^3H]-serotonin in the presence of iproniazide to prevent 5-HT metabolism. Examination of these lungs with the electron microscope failed to reveal ultrastructural abnormalities at the level of the alveolar septum. On autoradiography of the O_2 -exposed lungs, silver grains were localized predominately to the endothelial cells (Fig. 6 and Table 3). These results indicate that this degree of hyperoxia did not cause ultrastructural damage to the lung and that the accumulation of serotonin even with oxygen-exposed animals was still predominately in the endothelial compartment.

MECHANISMS OF DEPRESSION OF AMINE UPTAKE

Clearance of serotonin and norepinephrine was inhibited at an early stage in the development of pulmonary oxygen toxicity. The evidence that O_2 toxicity was at an early stage was the failure to demonstrate pulmonary derangement by morphologic, physiologic, or metabolic criteria. On the other hand, removal of imipramine was not depressed at this stage of oxygen exposure. This differential effect on clearance can be explained by the differences in normal handling of the amines. Serotonin and norepinephrine are probably transported actively by the pulmonary endothelial cell while imipramine is cleared by passive binding. Therefore, we conclude that oxygen may exert its effect on the pulmonary endothelial cell membrane and thereby interfere with the active transport process for circulating amines. Since uptake of both serotonin and norepinephrine was affected, the toxic effect of O_2 either involved more than one carrier or some basic mechanism common to the transport of both amines. The results with metabolic studies suggest that the common mechanism was not alteration of energy supply. The answer to this problem awaits further definition of the precise mechanisms involved in amine transport.

SUMMARY AND CONCLUSIONS

Exposure to elevated partial pressure of oxygen results in early and reversible depression of active amine (serotonin and norepinephrine) transport by the rat lung. A possible mechanism is that O_2 exerts a toxic effect on the pulmonary endothelial cell membrane. The findings may provide a convenient metabolic marker for the early toxic effects of oxygen on the lung. The significance of decreased amine clearance in the pathogenesis of the systemic manifestations of oxygen poisoning remains to be evaluated.

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DISCUSSION

PARTICIPANT: Is there any speculation as to why the process for serotonin is so different from that for prostaglandin? As you reiterated, for prostaglandins, the rate-limiting step is not uptake but metabolism by the dehydrogenase.

DR. FISHER: Why is uptake rate-limiting for serotonin but metabolism rate-limiting for prostaglandin? Is that right? I suppose that it relates to the respective rate constants for the processes. The serotonin uptake process is high capacity (high V_{max}) with a relatively low K_m compared to the MAO. The reverse, I suppose, is true for the prostaglandins. In addition, there's probably intracellular binding of serotonin, because it doesn't leak out. Once it's transported in, it will stay there for a long time. For prostaglandins, that apparently is not true from what Tom Eling said. In the absence of metabolism, prostaglandin leaks out. That may also play a role.

PARTICIPANT: Have you and, say, Tom Eling ever exchanged transport inhibitors?

DR. FISHER: I have not used his.

PARTICIPANT: I wonder if you've ever given thought to the hypothesis that has been advanced that cell death is responsible for the altered lung function.

DR. FISHER: I suppose that if there were cell death in oxygen toxicity, then that would lead to functional alterations. The LD_{50} for rats in oxygen is about 72 hours. Morphologic and physiologic changes probably take roughly 48 hours to reach the stage where you might see cell death. We think that we were looking at an earlier stage. In addition, the effects were rapidly reversible. I don't think that cell death accounts for what we found. We think that these effects precede morphologic or physiologic change and represent an early stage. Certainly, if the oxygen exposure were continued, then you'd begin to see things like cell death as well as pulmonary edema which then might cause nonspecific alterations in nonrespiratory lung functions.

Session VII:
Summary and Future Research Needs

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Summary and Future Research Needs

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DR. BROMBERG: It will be hard to summarize this conference in the 15 minutes that we each have, and I don't think either of us is going to make a formal attempt to do so. First, I can speak for all of us in thanking the EPA for arranging this symposium and for inviting a very distinguished panel of articulate and ingenious scientists. Secondly, I want to point out that the EPA has remarkable powers. Having put out the lure of a meeting at Hilton Head Island in the middle of the cold winter, and having gotten everyone down here on a beautiful Sunday, they proceeded to change the weather to keep everyone indoors and in perfect attendance. It would be awfully nice if they could call upstairs and switch it all back again, so that some of us, or all of us perhaps, could get home.

This is a remarkable meeting in that, with very few exceptions, almost no mention was made of pulmonary mechanics and of gas exchange. That doesn't happen very often in meetings devoted primarily to lung research, but perhaps has been happening more frequently in the recent past and it may become a trend perhaps for the future. Another interesting note was that almost everyone is working or collaborating with an ultrastructuralist, or would like to have access to ultrastructural techniques. Dr. Reid, among others, gave us a very nice demonstration of how ultrastructural techniques can be combined with biochemical techniques in experimental studies of the reaction of the lung to insults. Dr. Satir presented most elegant ultrastructural biochemical data on the mechanism of ciliary motion.

The organization of the meeting, as I perceive it, looks at the lung as having three segments. There was a large area of attention to the epithelium,

and at the end, there was a large area of attention to the endothelium. In the middle, I suppose appropriately, there were a number of speakers who talked about cells that more or less fit between the epithelium and the endothelium. Notably missing, or in large measure missing, were discussions of smooth muscle and of neural function at more than a passing level. The keynote of the epithelial sections was set by Dr. Boucher and Dr. Gatzky, who pointed to the barrier function of the epithelium and the attempts to quantitate the nature and the site of this barrier. They paid special attention to the intercellular junctions and the so-called tight junctions of the epithelium and defined their properties by electrical measurements and by using a variety of non-charged probe molecules.

Of particular importance are the macromolecules which may gain access to important cells residing deep in the epithelium. These cells were discussed by Dr. Bienenstock, Dr. Brain, and Dr. Wasserman. I might parenthetically add that there are a few lucky M.D.s who have picked up a Ph.D. at this meeting, and that's another sign of the power of the Environmental Protection Agency. Dr. Wasserman is among them, and I think Dr. Fisher also is a beneficiary. In any event, these powerful cells--Dr. Spitznagel's cell, the poly, the mast cell of Dr. Wasserman, and the "big Mac" of Dr. Brain--have an enormous armamentarium. It's important to consider how materials deposited in the airway may or may not be able to gain access to these cells, and how environmental pollutants and toxic agents might alter the barrier function of the epithelium and permit more ready access of other inhaled materials to these critical cells, which when turned on have the potential not only for protective action, but apparently for very damaging actions. We were shown by Dr. Wasserman in particular, and other speakers as well, the remarkable balance between active effects and mechanisms which repair or inhibit these effects.

This underlies a big problem that the EPA faces in trying to develop its research program. It may be relatively easy to show effects of one sort or another, but at what point do these effects go from the normal range or the range that can be dealt with by intrinsic mechanisms to the point where you have disease. To do this, one must bear in mind the intrinsic variability of the human subject. The fact that some of us have less hair than others and some of us have brown eyes while others have blue eyes is obvious. But there are many other differences of which we're perhaps less aware that make some of us more susceptible to certain insults than others, because our compensatory mechanisms are not as effective. A stress that can be easily tolerated by one individual may become a stress that produces overt disease in another. Even though the EPA is a regulatory agency and will continue to look on its role in research as developing data that directly abut on its regulatory function, the Agency must bear in mind that it is important to understand mechanisms to the extent that one can predict idiosyncrasies and unusual reactions that some

people in the population will have to a given stress. To do this "blindly" using empirical protocols is impossible. There are too many agents to be tested and too many possible protocols. To do well-directed, intelligent, pertinent experiments, one has to have some reasonable conceptions of mechanism.

Another problem that the EPA in particular has to face is that the payoff is human effects. Yet, the experiments that can be performed in man are limited. In the past few days scientists have described to us systems that are very complex and far removed from the intact human being. But there are other techniques that are either directly applicable or potentially applicable to intact human subjects. We should make efforts to develop techniques that can be used in human subjects, and to apply them. For example, in studying tracheal tissue one can look at rats and hamsters, and so forth, but it is also possible with a fiberoptic bronchoscope to obtain samples of tracheal epithelium. Such epithelium can be cultured successfully. Dr. Collier has done so by using specimens that we have provided for him during ordinary bronchoscopies in clinical practice. He has been able to show by using such materials that these specimens can be maintained for many days, that they retain ciliary activity, that they can be infected by a variety of specific infectious agents, that characteristic morphologic pictures can be produced, and that these pictures are different from what is seen when one uses the same infectious agents in animal models. It's going to be important to try as much as possible to use human tissue, even for in vitro experiments. Dr. Boucher has shown us techniques that can be applied to intact animals, and a few steps have been made to apply them to intact humans, in whom one can measure one of the bioelectric parameters of the airways epithelium, and one can even induce local changes by the local application of very minute quantities of drugs. Other examples could be given. We always need to bear in mind the potential application of our techniques to human studies and the use of human tissue as much as possible in our experiments.

This is a very exciting era in lung biology; people with all kinds of background and training have been stimulated in a variety of ways to look at the lung as more than an organ that simply deals with shuttling air in and out, and transferring oxygen to the blood, and CO₂ out of the blood. I foresee that this kind of research is going to be of increasing importance to all aspects of government activity, to the NIH, NIEHS, and also to the EPA. It will take some ingenuity to do the right kind of experiments certainly, but it is going to take an attitude on the part of the EPA, in particular, not to disregard mechanism in favor of what may seem to be the short-term payoff of defining levels that are or are not "toxic." To do the latter job well will require some very clever application of the knowledge of mechanisms, and that is the plea that I would like to leave with you. Thank you.

DR. MENZEL: We've seen here the beginnings of a new era in pulmonary research. It is directed to the definition of the normal physiologic state of the lung, emphasizing both respiratory and nonrespiratory metabolism. In defining the pathophysiologic state, mechanisms of action must be known so that alterations in particular values may be recognized as abnormal. Since biochemical measurements often are more sensitive than morphologic changes, connections between altered morphology and altered physiology must be sought. Unfortunately, the biology of the lung is but poorly understood. Much effort, then, is being expended on basic or baseline data to increase our surety that the measured effect on exposure to an environmental pollutant is indeed abnormal and hence toxic. To these ends our tools are still blunt and need to be honed to as fine an edge as possible.

Controversy surrounds the use of animal data in assessing human toxicity. Much of this discord results from the pioneer state of lung research. To be sure, there are differences between man and animals, especially in the morphology of the lung. But there are far fewer differences in the basic physiologic processes as seen here for ion transport, metabolism of xenobiotic compounds, and the uptake and metabolism of prostaglandins, angiotensin, and biogenic amines. On this scale the difference between animals and man is one of dose.

Morphology is highly important, as evidenced by the heavy collaboration between physiologists, pharmacologists, and morphologists. One approach has been to eliminate morphology by studying pure systems. Here the biochemistries of the hepatic and pulmonary systems of cytochrome P₄₅₀, for example, are remarkably similar. Tissue specificity is expressed in the molecular forms of cytochrome P₄₅₀ present in the lung and the differences in inducibility by environmental agents. The uptake mechanisms unique to the lung are likely, however, to encourage recycling and higher concentrations of reactive intermediaries than in the liver. This may make the lung more susceptible to both environmental carcinogens and toxicants acting through activated intermediaries which bind covalently to cell macromolecules. The biochemistry and pharmacology of the lung has been aided by the isolation and culture of both organs and specific cells. Much more work is needed here to identify susceptible lung cells and to develop specific markers, enzymatic or glycoprotein, indicative of pulmonary damage. Release of marker enzymes by the liver and heart has been established as a valuable tool for estimating hepatic damage by toxicants and myocardial damage by ischemia in the clinical assessment of man. Similar techniques are likely for pulmonary damage.

The development of ex vivo and in vivo perfused lung preparations has been accomplished in the last five years. It is now possible to study the transient events of prostaglandin metabolism which had been obscured by competing factors and compensatory mechanisms in intact preparations. Of particular note are

the studies relating SO_2 to bronchitis and the consequences of aberrant prostaglandin metabolism in asthmatics. Perhaps here we will understand some of the more subtle effects seen in man on lifetime exposure to polluted air.

We cannot lose sight of the fact that regulation of pollution is the ultimate aim to which these studies will be applied. Therefore, it is important to define the point at which an effect ceases to become a normal response and becomes pathophysiologic. It is also necessary to attempt to extrapolate these measurements made in animals to the human condition in a manner which is defensible and at the same time sensitive. Certainly the question of the variability of man in regard to his response is particularly important. This integration is represented by the organization of this meeting. It represents the philosophy of those within the Health Effects Research Laboratory with whom I have had the pleasure to work, starting with Dr. Gordon Hueter, Director of HERL, and Dr. William Durham who are not here today but are represented most immediately, in our presence, by Dr. D. E. Gardner and Dr. Ed Hu and their colleagues. Certainly, they recognize the need for the development of methods that are sensitive, the application of new methods, and the integration of these to toxic exposures which are relevant to the exposure of man in the environment and ultimately to the prevention of disease. This particular mission is deserving of the finest research that we can bring to the issue. I am very pleased to have been able to associate with you in demonstrating that it is possible to bring together high quality, thesis-directed research in the application to inhalation toxicology. Thank you.